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Institute of Translational medicine  
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**Wild-type N-Ras complements mutant K-Ras in  
pancreatic cancer cell lines but K-Ras has a specific  
role in cell cycle independent regulation of G2 cyclins**

Thesis submitted in accordance with the requirements of the University of Liverpool for  
the degree of Doctor in Philosophy

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**April 2015**

## **Declaration**

I declare this thesis is based on the results of my own work and wherever I have incorporated the work of others it has been clearly stated.

This work has not been submitted for any other degree nor is it in the process of being submitted for any other degree, either at the University of Liverpool or anywhere else.

## Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) is nearly always associated with mutant K-Ras. Nevertheless, targeting oncogenic K-Ras has so far proved ineffective in treating this form of cancer and pancreatic cancer cell lines can become K-Ras independent. Other forms of Ras are rarely mutated but wild type N-Ras and H-Ras have been shown to be present alongside functional K-Ras mutations and have been demonstrated to increase responsiveness to growth factors. Beyond this little evidence had previously been gathered on the activity or function of N-Ras and H-Ras in PDAC. Therefore, this thesis aimed to determine if other Ras isoforms are active in PDAC cell lines and what effect they may have on controlling cell division, oxidative metabolism, cytokine expression and the phospholipid composition of the membrane.

The presence of active N-Ras and K-Ras was identified in three of the four human PDAC cell lines tested. Only active K-Ras was detected in a cell line derived from a mouse model of pancreatic cancer driven by heterologous expression of mutant *KRAS*. N-Ras was shown to be functioning alongside K-Ras to control the relative level of oxidative metabolism in the Suit-2 and a faster growing variant of the Panc-1 cell lines, but K-Ras acts alone in the slow growing Panc-1 cell line that does not contain N-Ras. N-Ras and K-Ras were shown to have different effects on the levels of cytokines, although K-Ras is largely independent of N-Ras in its regulation of phospholipid composition.

A novel N-Ras independent mechanism for K-Ras transcriptional control of cyclin B1 was demonstrated. When K-Ras is depleted cyclin B1 and cyclin A are decreased. Cyclin B1 transcription can be rescued by inhibition of the Proteasome. A model is proposed whereby an unknown protein or proteins activates cyclin B1 transcription in a cell-cycle independent fashion and is protected from proteasomal degradation by K-Ras.

These results suggest that mutant K-Ras can act in conjunction with wild-type N-Ras, but also can function independently to regulate G2 cyclins.

## Acknowledgements

I would like to thank my supervisors Dr Costello and Dr Greenhalf for providing me with the opportunity to work in their lab and providing me with guidance and supervision as my project has evolved.

A special thank you has to go to Dr Victoria Shaw, Dr Jane Armstrong, Dr Asmaa Salman, Dr Karen Aughton, Dr Claire Jenkinson and Mrs Elizabeth Garner. Without your help I would not have been able to learn several of the more complex techniques that were used in my project. They have also been instrumental in the understanding of many of the novel and surprising results by being willing to discuss my ideas and providing input in interpretation of many of the results that I have found.

A special thanks goes to my family, Anna, and all of my friends whose support and belief in me have been instrumental in allowing me to achieve all I have.

It has been a pleasure to work in the Department, this was Surgery and Oncology when I started, headed by Professor John Neoptolemos who helped me a great deal and shared his enthusiasm for the pancreas with me, it later became Molecular and Clinical Cancer Medicine led by Professor Pettitt to whom I also owe thanks.

I was supported by the EU Framework 7 Cancercalia consortium; in particular Professor Johan Swinnen who kindly performed the phospholipid analysis reported here. Finally, I would like to thank the National Institute for Health Research Liverpool Pancreas Biomedical Research Unit, UK who along with Cancercalia provided the financial support without which this work would not have been possible.

## List of Abbreviations

SI units have been used unless indicated and standard gene symbols have been used except as indicated below.

Akt	RAC-alpha serine/threonine-protein kinase
AMPK	AMP-activated Protein Kinase
APC/c	Anaphase promoting complex/cyclosome
ATCC	American tissue culture collection
ATP	Adenosine Triphosphate
AP-1	Activator protein 1
BCA	Bicinchoninic acid assay
CAKs	Cdk-activating kinases
Cdc	Cell division cycle
<i>CDK</i>	Cyclin dependent kinase gene
Cdk	Cyclin dependent kinase protein
CDKN	Cyclin-dependent kinase inhibitor
Chk	Checkpoint Kinases
ChoK	Choline kinase

DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	DL-dithiothreitol
DUSP	Dual specificity phosphatase
ECAR	Extra cellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EdU	5-ethynyl-2'-deoxyuridine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transformation
EMT	Epithelial- mesenchymal transformation
Erk	Extracellular-signal-regulated kinases
FACS	Fluorescent-activated cell sorter
FBS	Foetal bovine serum
FCCP	carbonyl cyanide 4-(Trifluoromethoxy) phenylhydrazone
GAPs	GTPase activating proteins

GCLP	Good clinical laboratory practice
GDP	Guanosine 5' diphosphate
GEFs	Guanine nucleotide exchange factor
Grb2	Growth factor receptor-bound protein 2
GSK	Glycogen synthase kinase
GTP	Guanosine 5' triphosphate
<i>HRAS</i>	Harvey rat sarcoma viral oncogene homolog
H-Ras	Protein produced by Harvey rat sarcoma viral oncogene homolog
IL	Interleukin
IPMN	Intraductal papillary mucinous neoplasia
KPC	Kras <sup>LSL.G12D/+</sup> ; p53 <sup>R172H/+</sup> ; PdxCretg/+
<i>KRAS</i>	Kirsten rat sarcoma viral oncogene homolog
K-Ras	Protein produced by Kirsten rat sarcoma viral oncogene homolog
M	Mitosis
MAPK	Mitogen-activated protein kinases
MEK	Mitogen-activated protein kinase/Erk kinase
MRLC	Myosin regulatory light chain

Myt	Myelin transcription factor
<i>NRAS</i>	Neuroblastoma RAS viral oncogene homolog
N-Ras	Protein produced by Neuroblastoma RAS viral oncogene homolog
OCR	Oxygen consumption rate Neuroblastoma RAS viral oncogene homolog
PA	Phosphatidic acid
PAK1	P21 protein (Cdc42/Rac)-activated kinase 1
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered Saline
PBST	Phosphate buffered Saline with tween
PC	Phosphatidylcholine
PCho	Phosphocholine
PDAC	Pancreatic ductal adenocarcinoma
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PLD	Phospholipase D



PPP1R12C	Protein phosphatase 1 regulatory subunit 12C
pRB	Retinoblastoma protein
PS	Phosphatidylserine
Rab	Ras-like proteins Associated to the Brain
Raf	Rapidly accelerated fibrosarcoma
RalBP1	RalA binding protein 1
RalGDS	Ral guanine nucleotide dissociation stimulator
Rals	Ras-like GTPases
RhoA	Ras homolog gene family, member A
RISC	RNA-induced silencing complex
RKIP	Raf kinase inhibitory protein
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
RSK	Ribosomal s6 kinase
SC	Surviving cells
SCF	Skp1, cullin and the F-box
Shc	Src homology 2 domain (containing) transforming protein 1

Skp1	S-phase kinase-associated protein 1
<i>SMAD</i>	Mothers against decapentaplegic gene
Smad	Mothers against decapentaplegic protein
SOP	Standard operating procedure
Sos	Son of sevenless protein
<i>TP53</i>	Tumour protein p53
<i>YAP1</i>	Yes associated protein 1 gene
Zeb1	Zinc finger E-box-binding homebox 1

## Contents

CHAPTER 1:	Introduction.....	19
1.1	Pancreatic cancer .....	19
1.2	K-ras mutations occur early and their importance in pancreatic cancer.....	21
1.3	Structure and function of Ras isoforms .....	24
1.3.1	K-Ras as a molecular switch .....	24
1.3.2	Promoters of K-Ras activity.....	25
1.4	Downstream targets of K-Ras .....	27
1.4.1	MEK/Erk mediated signalling.....	28
1.4.2	PI3K mediated signalling.....	34
1.4.3	Ral GTPases .....	35
1.4.4	Myc .....	36
1.5	Phosphatidylcholine as a lipid membrane component. ....	37
1.6	K-Ras as a target for treatment.....	39
1.7	Cell cycle .....	41
1.7.1	Overview .....	41
1.7.2	Control of the cell cycle .....	42
1.8	Myc involvement in the cell cycle.....	51
1.9	AIMS .....	53
CHAPTER 2:	Materials and Methods.....	54
2.1	Cell culture .....	65

2.2	Harvesting of cells for optimisation of western blot .....	65
2.3	Harvesting of cells following siRNA treatment for western blot .....	65
2.4	Preparation of cell lysates for western blot .....	66
2.5	Quantification of total protein using the Bicinchoninic acid assay .....	66
2.6	Western blot.....	67
2.7	siRNA treatment of cell lines .....	69
2.8	Growth curve experiments .....	71
2.8.1	Harvesting and testing cell viability.....	71
2.9	FACS analysis of samples .....	72
2.9.1	Propidium iodide treatment of cells for cell cycle analysis .....	72
2.9.2	S-phase labelling of cells using EdU .....	74
2.10	Preparation of cells for analysis by the Seahorse Bioanalyser .....	75
2.11	Preparation of cells for lipid analysis .....	77
2.12	Pulldown assays .....	77
2.12.1	Active Ras pulldown .....	77
2.12.2	Ubiquitin pulldown .....	78
2.13	Transcriptional analysis .....	79
2.13.1	Preparation and harvesting of samples.....	79
2.13.2	Preparation of RNA.....	80
2.13.3	Preparation of cDNA.....	80
2.13.4	qPCR .....	82

2.14	Measurement of cytokines .....	83
2.15	Statistical methods .....	84
CHAPTER 3: Results 1: Activity and Functions of RAS isoforms.....		85
3.1	K-Ras can be depleted in pancreatic cancer cell Lines. ....	85
3.2	K-Ras depletion results in viable cells and protein levels will return to normal several days after siRNA treatment.....	85
3.3	Active Ras isoforms in pancreatic cancer cell lines .....	91
3.4	N-Ras functions in human pancreatic cancer cell lines.....	93
3.4.1	Ras isoforms modifying growth patterns .....	93
3.4.2	Ras isoforms independently alter cytokine expression patterns .....	98
3.4.3	Membrane lipid compositions are modified uniquely by K-Ras and N-Ras .....	101
3.5	Summary.....	112
CHAPTER 4: Results 2: K-Ras modulation of G2 cyclins.....		113
4.1	Depleting K-Ras but not N-Ras results in a change in the expression pattern of G2 cyclins.....	113
4.2	Effect that depletion of K-ras and N-Ras has on other targets of the APC/c complex .....	114
4.3	FACS analysis of Suit-2 cells when K-Ras is depleted.....	115
4.3.1	Propidium Iodide analysis.....	115
4.3.2	EdU FACS analysis.....	116

4.4	Is MAPK or PI3K/Akt signalling responsible for depletion of cyclins?.....	119
4.5	FACS time course comparing K-Ras and cyclin D knockdowns.....	123
4.6	Potential intermediates between K-Ras and the G2 cyclins .....	123
4.6.1	AMPK .....	123
4.6.2	Ral A and Ral B .....	126
4.7	Bortezomib treatment of K-Ras depleted cells.....	127
4.8	FACS analysis and western blots to determine if G2 cyclins are present in G1 .....	130
4.9	Transcriptional analysis of G2 cyclins when K-Ras is depleted .....	132
4.10	Effect of Bortezomib on the levels of G2 cyclin transcripts when K-Ras is depleted .....	133
4.11	Summary .....	135
CHAPTER 5:	Discussion .....	136
5.1	K-Ras depletion results in viable cells .....	136
5.2	Active Ras isoforms in pancreatic cancer cells .....	137
5.3	Effects of Ras on the cyclins .....	141
5.4	Future work .....	145
CHAPTER 6:	References.....	147

## List of Figures

Figure 1-1 : Diagram of the original six hallmarks of cancer .....	20
Figure 1-2 : K-Ras signalling pathways.....	27
Figure 1-3 : Diagram of the Raf/MEK/Erk signalling pathway.....	33
Figure 1-4 : Diagram showing the synthesis of Phosphatidylcholine from PCho via the CDP-choline Kennedy pathway.....	40
Figure 1-5 : Diagram demonstrating the phases of the cell cycle.. ..	41
Figure 1-6 : Diagram showing how multiple phosphorylation events are required to fully activate cyclin A and E transcription.....	49
Figure 2-1 : Example western blot demonstrating how size markers were compared to the lanes of interest.....	68
Figure 3-1 : K-Ras depletion in pancreatic cancer cell lines. ....	87
Figure 3-2 : Graphs showing viability of Suit-2 cells.....	88
Figure 3-3 : Western blot of Suit-2 cell time course demonstrating the level of K-Ras protein up to 216 h post siRNA treatment. ....	89
Figure 3-4 : Cell numbers 48 hours 144 hours and 192 hours following K-Ras depletion .....	90
Figure 3-5 : Active Ras in Suit-2 cell lines.....	91
Figure 3-6 : Total and active Ras isoforms in Suit-2 cells.....	93
Figure 3-7 : Total and active Ras isoforms in pancreatic cancer cell lines. ....	94
Figure 3-8 : Effect of K-Ras and N-Ras on oxidative phosphorylation in Suit-2 cells. ....	95
Figure 3-9 : Active N-Ras presence in two different Panc-1 cell lines.....	96
Figure 3-10 : Aerobic stress test of two Panc-1 cell lines with different dividing times. ....	97

Figure 3-11 : Effect of K-Ras and N-Ras on the cytokine expression profile of Suit-2 cells. ....	100
Figure 3-12 : Membrane lipid analysis of phosphatidylcholine.....	104
Figure 3-13 : Membrane lipid analysis phosphatidylethanolamine. ....	105
Figure 3-14 : Membrane lipid analysis phosphatidylinositol.....	106
Figure 3-15 : Membrane lipid analysis phosphatidylserine. ....	107
Figure 3-16 : Membrane lipid analysis of phosphatidylcholine levels when cells are treated with MAPK and PI3K inhibitors.....	108
Figure 3-17 : Membrane lipid analysis of phosphatidylethanolamine levels when cells are treated with MAPK and PI3K inhibitors.....	109
Figure 3-18 : Membrane lipid analysis of phosphatidylinositol levels when cells are treated with MAPK and PI3K inhibitors.....	110
Figure 3-19 : Membrane lipid analysis of phosphatidylserine levels when cells are treated with MAPK and PI3K inhibitors.....	111
Figure 4-1 : Cyclin levels when K or N-Ras were depleted in Suit-2 cells. ....	114
Figure 4-2 : Levels of proteins targeted by the APC/c after K or N-Ras are depleted..	115
Figure 4-3 : FACS analysis following K-Ras depletion. ....	116
Figure 4-4 : EdU cell cycle analysis of K-Ras depleted Suit-2 cells.. ....	117
Figure 4-5 : Analysis of EdU labelling over time. ....	119
Figure 4-6 : Effect of MAPK and Akt inhibitors on cyclin levels when K-Ras is depleted.. ....	121
Figure 4-7 : Effect of combined treatment of MAPK and Akt inhibitors on cyclin levels when K-Ras is depleted.....	122



Figure 4-8 : Comparison of the effects of both K-Ras and cyclin D depletion on the cell cycle and G2 cyclins. ....	125
Figure 4-9 : PhosphoAMPK and how its levels change in response to K-Ras depletion.. ....	126
Figure 4-10 : Depletion of Rals and its effect the levels of G2 cyclins. ....	127
Figure 4-11 : K-Ras dependent increase in G2 cyclin level is not the result of blocking proteolysis of ubiquitinated cyclins. ....	129
Figure 4-12 : Analysis to determine the presence of G2 cyclins in cells predominately in G1 phase cells. ....	131
Figure 4-13 : Effect of depleting K-Ras or cyclin D on G2 cyclin transcript levels....	133
Figure 4-14 : Effects on G2 cyclins when Suit-2 cells are treated with K-Ras siRNA and bortezomib.....	134
Figure 5-1 : Model suggesting a mechanism to explain non cell cycle dependent G2 cyclin levels.....	145

## List of Tables

Table 1: Table showing density that cells were seeded at in each well of a six well plate for siRNA experiments .....	69
Table 2: Concentrations of siRNA used to treat cells seeded in a six well plate. ....	70
Table 3: Controls used for siRNA experiments .....	70
Table 4: Reaction mixture for EdU staining. ....	75
Table 5: Number of cells to be seeded per well in a 24 well Seahorse Bioanalyser plate .....	76
Table 6: Concentration and volume of drugs loaded into the injection ports of the Seahorse Bioanalyser .....	76
Table 7: Recipe for preparing the sample for DNA wipe-out treatment.....	81
Table 8: Table showing the recipe for preparing the reverse transcriptase reaction.....	81
Table 9: Recipe for preparing qPCR samples .....	82
Table 10: PCR protocol used for Real-Time PCR. ....	83
Table 11: Table showing cytokines in the Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay kit. ....	99

## CHAPTER 1: INTRODUCTION

### 1.1 PANCREATIC CANCER

Cancer is a common disease that can present in many different forms and it has been defined using six hallmarks that are traditionally thought to be the processes that require modification for cancer formation (Figure 1-1). (Hanahan and Weinberg, 2000) More recently two additional hallmarks have been proposed; reprogramming energy metabolism and evading immune destruction.(Hanahan and Weinberg, 2011)

Pancreatic cancer is one of the deadliest cancers with a high mortality rate being common and Pancreatic Ductal Adenocarcinoma (PDAC) is the most common form. Pancreatic cancer was the 14<sup>th</sup> most common cancer in the USA (48,960 estimated cases) in 2014 but was the 6<sup>th</sup> highest cause of cancer related death (40,560 estimated deaths). (Siegel et al., 2015) In 2012 pancreatic cancer was the 5<sup>th</sup> most common cause of cancer related death in the UK with 8662 deaths. (UK, 2014) Patients diagnosed with pancreatic cancer have a median survival of just 6 months (Vincent et al., 2011) and currently chemotherapy has little effect, with surgery being the only possible curative intervention available. Even patients that undergo pancreatic head resection only have a 10-25% chance of 5 year survival. (Distler et al., 2013; Winter et al., 2006) Survival can be improved with addition of adjuvant therapy post-surgery, however some studies have reported that there was still a cohort of patients that will relapse following complete resection of the tumour. (Neoptolemos et al., 2009; Oettle et al., 2007)

There are several different non-invasive ductal lesions that may occur before a cancer (PDAC) arises. The most widely accepted form of progression is via intraductal proliferation called Pancreatic Intraepithelial Neoplasias (PanINs). (Hruban et al., 2001) PanINs are usually microscopic lesions that occur in the pancreatic ducts and can be classified into three differing grades of severity based on their atypical structures.(Hruban et al., 2004) In addition to PDAC, other forms of pancreatic disease exist and these can also have K-Ras mutations present. It is still unclear why the pancreas seems so exquisitely prone to this particular mutation or whether this has effects related specifically to pancreatic transformation. Intraductal Papillary Mucinous Neoplasms (IPMN) are a precursor to a non PDAC malignancy that, has also been shown to have mutant K-Ras present in 46% of cases.(Amato et al., 2014)

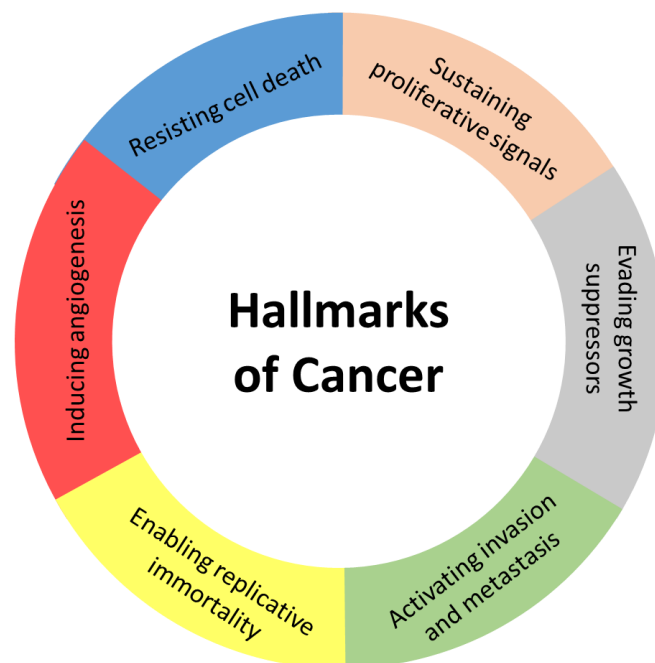


Figure 1-34: **Diagram of the original six hallmarks of cancer (adapted from Hanahan, *et al.*(Hanahan and Weinberg, 2011))**

## 1.2 K-RAS MUTATIONS OCCUR EARLY AND THEIR IMPORTANCE IN PANCREATIC CANCER

The Kirsten Ras onco-protein was first identified as being expressed from the Kirsten sarcoma virus. Homologues to the viral gene were found in the human genome, the first proving to be a pseudogene, while the 2<sup>nd</sup> is a proto-oncogene referred to as *KRAS*.(Chang et al., 1982) Mutation of the *KRAS* gene is typically recognised as an early occurrence in PanIN formation and is seen in many PDACs with estimations ranging from 52% (Kim et al., 2011) to 93%. (Biankin et al., 2012) Other Ras isoforms exist (*NRAS* and *HRAS*) but have not been investigated in any great detail because mutations have rarely been found (Biankin et al., 2012) in PDAC and their role has therefore, largely been ignored. Although, there is evidence to suggest that both N-Ras and H-Ras are present in pancreatic cancer cells (Omerovic et al., 2008) and that they can be responsible for an increase in phospho Erk levels in response to Epidermal Growth Factor (EGF) stimulation of MiaPaCa2 cells.(Young et al., 2013) Several other mutations such as in *SMAD4*, *TP53* and *CDKN2A* are also common and are therefore, assumed to be drivers, *KRAS* mutation is often thought of as a key early event for PDAC formation. Indeed the activation of the *KRAS* gene under its own promoter in pancreatic progenitor cells has been shown to be sufficient to cause the formation of pancreatic cancer in the mouse. (Hingorani et al., 2003) The developmental process in the mouse is similar to that normally observed in humans because there are PanIN like lesions, which appear before cancer formation. (Hingorani et al., 2003) The pancreatic progenitor cells are able to form islet cells, acinar cells and ductal epithelia. Switching on mutant K-Ras in these progenitor cells is sufficient to cause PanIN formation later in the life of the mouse and these can form tumours. The rate of formation of both PanINs

and tumours is greatly enhanced if the mice are crossed with mice engineered to express mutant p53 protein (known as KPC mice). (Hingorani et al., 2003)

When K-Ras alone is mutated in the already matured acinar (Grippe et al., 2003) or ductal (Brembeck et al., 2003) cells, PanIN formation and subsequently cancer are not observed. The mutant K-Ras expressed under its own promoter that leads to PanIN has a stop codon that prevents production of the protein until it is looped out following Cre recombinase expression. The Cre is controlled by an acinar specific elastase promoter ensuring pancreas specific expression of the mutant protein and hence pancreatic tumours.(Hingorani et al., 2003) This only can occur however, if K-Ras is expressed during development or following cerulein induced pancreatitis if the cells have already differentiated (adult mice). (Guerra et al., 2007) This demonstrates the possibility that not only is the activation of oncogenic K-Ras required when cells have differentiated, but that they also require further non-genetic events such as tissue damage, or an inflammatory response to induce the formation of cancer.

Loss of mutant K-Ras after the formation of the tumour in mice has been demonstrated to result in rapid regression of the tumour (4-5 weeks) due to apoptosis.(Viale et al., 2014) However these tumours would rapidly reappear when oncogenic K-Ras was restored. They could also sporadically reoccur 4-5 months later when oncogenic K-Ras remained switched off. (Viale et al., 2014) When examined further Viale *et al* noted the presence of a subpopulation of surviving cells (SC) that were repopulating the tumour. When isolated and transplanted into mice, these surviving cells had a greater potential to form a tumour than the K-Ras dependent cells. Ablation of K-Ras signalling by pharmacological inhibition of MEK and PI3K pathways, similarly reduced tumour formation with recurrence occurring. (Viale et al., 2014) The SC demonstrated similar

characteristics to those produced by loss of the oncogene were observed when MEK and PI3K pathways were inhibited. (Viale et al., 2014) Therefore, it is suggested that in the heterozygous mouse tumours different subpopulations of cells exist with differing levels of addiction to K-Ras and some of these populations may in fact require K-Ras for survival. Furthermore, mutations such as the ones found in *YAP1* are beginning to be identified as possible compensatory mutations that can negate the loss of K-Ras and therefore overcome K-Ras addiction. (Kapoor et al., 2014; Shao et al., 2014)

This phenomenon of K-Ras addiction has also been observed in human cell lines, (Singh et al., 2009) where some cells have been shown to be addicted and therefore would undergo apoptosis when K-Ras is depleted. However other cell lines that are defined as independent, would not undergo apoptosis although their proliferation may be restricted. (Singh et al., 2009) This addiction appears to be related to Epithelial-Mesenchymal Transformation (EMT) as cells with a more epithelial phenotype tended to have a greater addiction to K-Ras, than cells that presented a more mesenchymal phenotype. (Singh et al., 2009) Additionally treatment of K-Ras dependent lung cancer cell line H358 with transforming growth factor  $\beta$ 1 to make them undergo EMT (measured by a loss of E-cadherin) resulted in decreased K-Ras addiction. Conversely depletion of *Zeb1* in Panc-1 cells (K-Ras independent) resulted in a more epithelial phenotype and causes an increase in K-Ras addiction. (Singh et al., 2009) Gene expression patterns of cell lines that were addicted or independent of K-Ras were then used to create an expression profile to predict if other cell lines will be addicted to K-Ras. The understanding of K-Ras addiction could have important clinical significance because as of yet, targeting K-Ras in the clinic has not proved successful. (Macdonald

et al., 2005) The existence of subpopulations of tumour cells that survive loss of K-Ras could be potentially responsible.

### 1.3 STRUCTURE AND FUNCTION OF RAS ISOFORMS

#### 1.3.1 **K-RAS AS A MOLECULAR SWITCH**

Ras isoforms can fine tune signals to many different downstream pathways, and K-Ras in pancreatic cancer has been shown to be involved in controlling multiple cellular functions such as; apoptosis, differentiation, migration and proliferation. (Spaargaren et al., 1995) K-Ras in its normal form is a GTPase that acts as a molecular switch. It is active when Guanosine 5' Triphosphate (GTP) is bound in the binding pocket and inactive when Guanosine 5' Diphosphate (GDP) is bound. Although K-Ras is a binary switch it has a gradient like response to activating signalling molecules. (di Magliano and Logsdon, 2013) The binding of GTP to Ras molecules is regulated by guanine nucleotide exchange factors (GEFs), which promote the binding of GTP by destabilising the nucleotide binding cleft. This destabilisation results in GDP leaving the binding site leaving it available for GTP binding. (Vetter and Wittinghofer, 2001)

K-Ras alone has a limited ability to hydrolyse GTP to GDP. However, this ability can be increased by interactions with specific GTPase activating proteins (GAPs). (Cherfils and Chardin, 1999; di Magliano and Logsdon, 2013) Specific point mutations in *KRAS* result in K-Ras being unable to effectively interact with GAPs, which in turn hinders the conversion of GTP to GDP. (Scheffzek et al., 1997) Failure of K-Ras to return to the inactive state may result in inappropriate signalling, which in turn will cause downstream pathways to be incorrectly activated/deactivated. GTP binding to K-Ras is not sufficient to measure effect of K-Ras on downstream targets as subcellular



localisation is also important; active K-Ras is bound to the cytoplasmic membrane. (Campbell et al., 2006) Additionally, evaluation of the mutant *KRAS* gene as a biomarker, showed that numerous healthy individuals had mutant K-Ras containing cells: including in the lung, pancreas (Lu et al., 2002; Parsons and Meng, 2009; Yan et al., 2005) and colon. (Lu et al., 2002) An explanation for this is that as we age mutations are being acquired; including K-Ras mutations (Lu et al., 2002; Parsons and Meng, 2009; Yakubovskaya et al., 1995; Yan et al., 2005) but these do not leave the protein constitutively active. (Huang et al., 2014) This was shown using a mouse model that had a *KRAS* mutant version inserted at physiological levels (one of the two copies was mutant) in pancreas cells. The model demonstrated that only 2% of total K-Ras was active in the model. (Huang et al., 2014) If *KRAS* mutation alone was sufficient to cause K-Ras protein to be constitutively active, then approximately 50% of total K-Ras protein would be in the active form. Oncogenic K-Ras on its own cannot generate enough K-Ras activity, because the mutation results only in an inefficient return to the inactive GDP bound state, rather than an increase in activation. (Pylayeva-Gupta et al., 2011) The delay in inactivation will contribute to tumorigenesis because it will allow more time for other signals (resulting from other genetic or epigenetic changes) to act: maintaining an active K-Ras signal. (di Magliano and Logsdon, 2013)

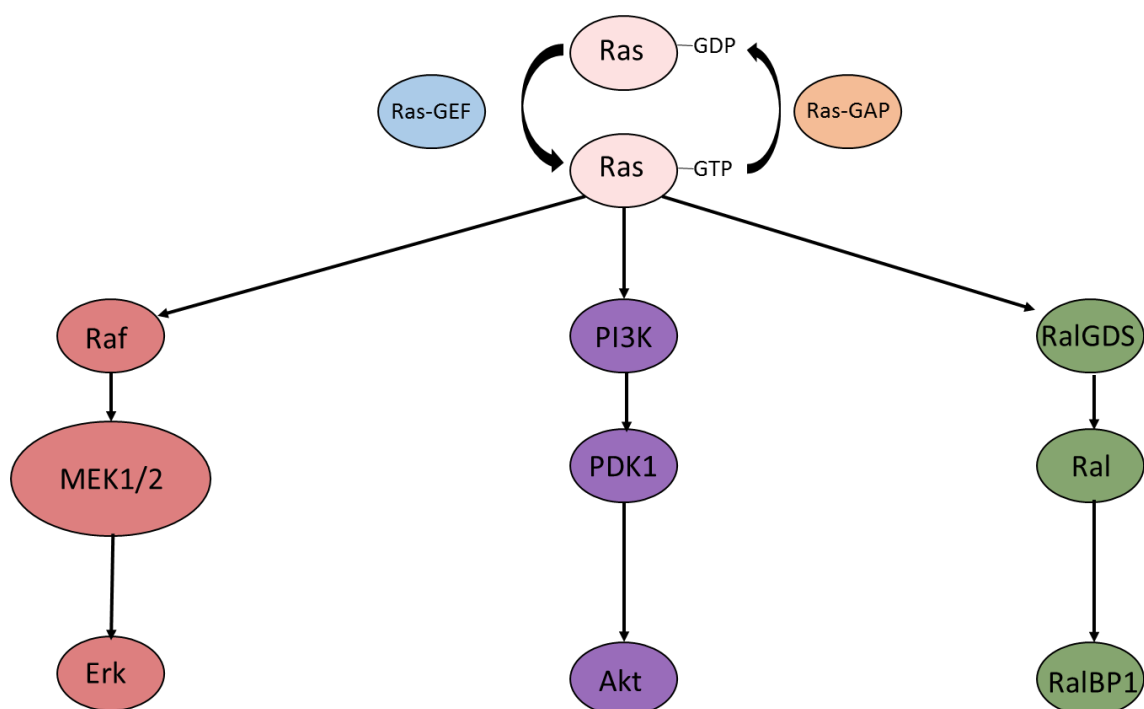
### 1.3.2 PROMOTERS OF K-RAS ACTIVITY

Epidermal Growth Factor Receptor (EGFR) has been shown to be involved in the progression of PDAC from its pre-neoplastic lesions. (Navas et al., 2012) This is in contrast to several other types of cancer where K-Ras mutations circumvent the need for EGFR. (Karapetis et al., 2008; Shigematsu et al., 2005) Activity of Erk and Akt (parts of the MAPK and PI3K pathways) increase when the cell is stimulated with EGF,

showing that mutant K-Ras alone is not adequate to activate downstream signalling pathways to the point of saturation. This is because despite K-Ras being constitutively active, EGF is still able to increase the levels of these downstream pathways. (Young et al., 2013) This perhaps indicates a role for wild-type Ras isoforms even in the presence of a mutant form. K-Ras appears to be able to make cells less sensitive to EGF signalling, as its loss results in an increase in the sensitivity to EGF. (Young et al., 2013) MAPK, which is activated by K-Ras, can negatively regulate EGF and its loss results in greater EGF sensitivity. One method by which K-Ras decreases EGFR sensitivity is via MAPK. (Young et al., 2013) Chemical inhibition of MEK prevents the phosphorylation of EGFR at residue p.T669. The inhibition of K-Ras is less efficient at preventing the phosphorylation than the chemical inhibition of MEK, but it does appear to increase EGFR signalling. (Young et al., 2013) Thus, other mechanisms of interaction between K-Ras and EGFR must exist. Ablation of EGFR prevents cerulean induced pancreatic fibrosis in K-Ras mutant mice. (Ardito et al., 2012) The loss of *Adam17* was also protective. The loss of Adam17 and EGFR results in a decrease in the overall levels of total and active Ras. This suggests that EGFR is able to increase the levels of active K-Ras above the threshold early in cancer development, which allows for formation of the cancer cell. (Ardito et al., 2012; Huang et al., 2014) The mutation of TP53 relieves the need for EGFR activation. (Navas et al., 2012) In mouse models where TP53 is mutated, EGFR inhibition is not an effective treatment, although it is still an effective preventative measure, by reducing the formation of tumours. (Ardito et al., 2012) Therefore, it is likely that EGFR is required for the initiation of tumour formation and its inhibition would be a good preventive strategy.

#### 1.4 DOWNSTREAM TARGETS OF K-RAS

K-Ras is known to be the mediator of signals for multiple different signalling pathways. (Figure 1-2) Mutation of *KRAS* has profound effects on: cell proliferation; survival; migration (di Magliano and Logsdon, 2013) and respiration. (Ying et al., 2012) The lack of efficient regimen targeting K-Ras directly has led to downstream targets becoming prime candidates for treatment.



Down stream effects resulting in modulation of multiple processes including: transcription of genes, growth, cell division, migration and cell cycle progression

**Figure 1-2: K-Ras signalling pathways. K-Ras is involved in the modulation of several different signalling pathways therefore, K-Ras is involved in many different cellular functions. Shown are some of the pathways that K-Ras is known to function through.**

#### 1.4.1 MEK/ERK MEDIATED SIGNALLING

The MEK/Erk signalling pathway allows extracellular signals to modify the transcription of various genes, or change the phosphorylation status of several key apoptotic proteins (McCubrey et al., 2007). (Figure 1-3) This allows the cells under normal conditions to adapt to their environment. Ras is required to be in the active form before it is able to bind to Raf. This typically occurs when growth factors, mitogens or cytokines bind to their receptor and activate the coupling complex Shc/Grb2/Sos. (McCubrey et al., 2007) The resultant complex will then promote the exchange of GDP for GTP on inactive Ras molecules, therefore activating them and allowing Ras to gather Raf at the membrane. (Yan et al., 1998) In addition to localisation to the membrane, several other events have to occur in order to activate Raf these are: dimerization of Raf, (Luo et al., 1996), loss of interaction with the Raf kinase inhibitory protein (RKIP), (Dhillon et al., 2002; Yeung et al., 1999), the association with several scaffolding complexes (Chang et al., 2003; Lee and McCubrey, 2002), and phosphorylation or dephosphorylation of specific Raf domains. (Fabian et al., 1993) Once activated, Raf proteins will activate MEK1 and MEK2 by directly phosphorylating them. (Coles and Shaw, 2002; Ramos, 2008) MEK is only fully activated after it has been phosphorylated by Raf and PAK1. (Frost et al., 1997; Frost et al., 1996) MEK1 and MEK2 are very specific and have no other known targets than the Erk proteins. (Neuzillet et al., 2014) MEKs will bind to Erks when they are inactive and keep them localised to the cytoplasm. (Fukuda et al., 1997) Once MEKs are activated they will phosphorylate Erks which results in their activation, and once active, MEKs release Erks allowing them to dimerise and translocate to the nucleus. (Ramos, 2008)

Active Erks in the nucleus or cytoplasm will bind to multiple different transcription factors, phosphatases and cytoskeletal proteins.

Erks are able to directly control the activity of transcription factors by phosphorylating them. This phosphorylation can be regulatory in several different ways. It can change their activity, binding affinity to DNA, subcellular localisation or the levels of protein present. (Yang et al., 2013) Activated Erk1 is able to phosphorylate Smad2 which stabilises the protein and increases the levels in the cell. Funaba *et al* demonstrates that this phosphorylation of Smad2 is required for maximal transcription of Smad2-dependant genes. The increase in Smad2 transcriptional activity is due to the protein being more stable, and therefore, being able to form a greater number of transcriptional activator complexes with Smad4. (Funaba et al., 2002)

The transcription factor Myc which targets include cyclin D, cyclin E, and E2F (Bretones et al., 2014) is also stabilised by phosphorylation by the MAPK pathway. Erk activation results in the phosphorylation of Ser 62 of the Myc protein stabilising it. (Sears et al., 2000) Another phosphorylation site at Thr 58 is targeted by glycogen synthase kinase (GSK-3) but this phosphorylation occurs after Ser62 has been phosphorylated. Thr 58 phosphorylation unlike Ser 62 results in the degradation of Myc. GSK-3 phosphorylation of this site can be prevented by PI3K signalling. (Sears et al., 2000) Therefore, two different downstream pathways of Ras are involved in the upregulation of the Myc transcription factor. K-Ras via MAPK signalling is also able to upregulate the transcription of cyclin D1 by stimulating the expression of activator protein 1 (AP-1) transcription factors. AP-1 can bind to the AP-1 binding site in the promoter region of cyclin D and stimulate its transcription. (Klein and Assoian, 2008).

Ras signalling via Erk is complex with feedback loops that can downregulate or upregulate Erk signalling. This is vital to ensure correct control of cell division and metabolism. (Owens and Keyse, 2007; Ramos, 2008) Erk can downregulate MEK directly by phosphorylating it, preventing MEK from being able to interact with PAK1. Thus removing PAK1's ability to enhance MEK's activation of Erk. (Slack-Davis et al., 2003) Phosphorylation of Raf at multiple sites by Erk prevents it being able to interact with Ras. (Dougherty et al., 2005) The inability of Raf to interact with Ras stimulates the dephosphorylation of the Ras bound GTP to GDP. (Ramos, 2008)

The Erk mediated phosphorylation of Sos was one of the first feedback loops in the Ras/Raf/MEK/Erk pathway to be discovered. (Corbalan-Garcia et al., 1996) Sos is a guanine nucleotide exchange factor (GEF) that interacts with Ras to activate it. The phosphorylation of Sos prevents its localisation to the plasma membrane where it normally interacts with Ras. The phosphorylation of Sos is not by Erk directly, but is mediated via RSK2 (Douville and Downward, 1997) which is activated by Erk directly in the cytoplasm. (Hauge and Frodin, 2006)

In the cytoplasm, Erk phosphorylates DUSP6 targeting it for degradation via the proteasome. (Marchetti et al., 2005) This acts as a positive feedback loop as phosphorylation of DUSP6 prevents it from being able to dephosphorylate Erk, leaving Erk active. (Muda et al., 1996) In contrast in the nucleus, phosphorylation of DUSP1 by Erk stabilises it, creating a negative feedback loop. (Brondello et al., 1997; Brondello et al., 1999) The regulation of DUSPs by Erk therefore will vary depending on where in the cell the phosphorylation is occurring. Localisation of Erk within the cell is an important aspect of cell signalling. Phosphorylation of Erk by MEK followed by its release (MEK binds to inactive Erk) allows for Erk dimerization, and is a key

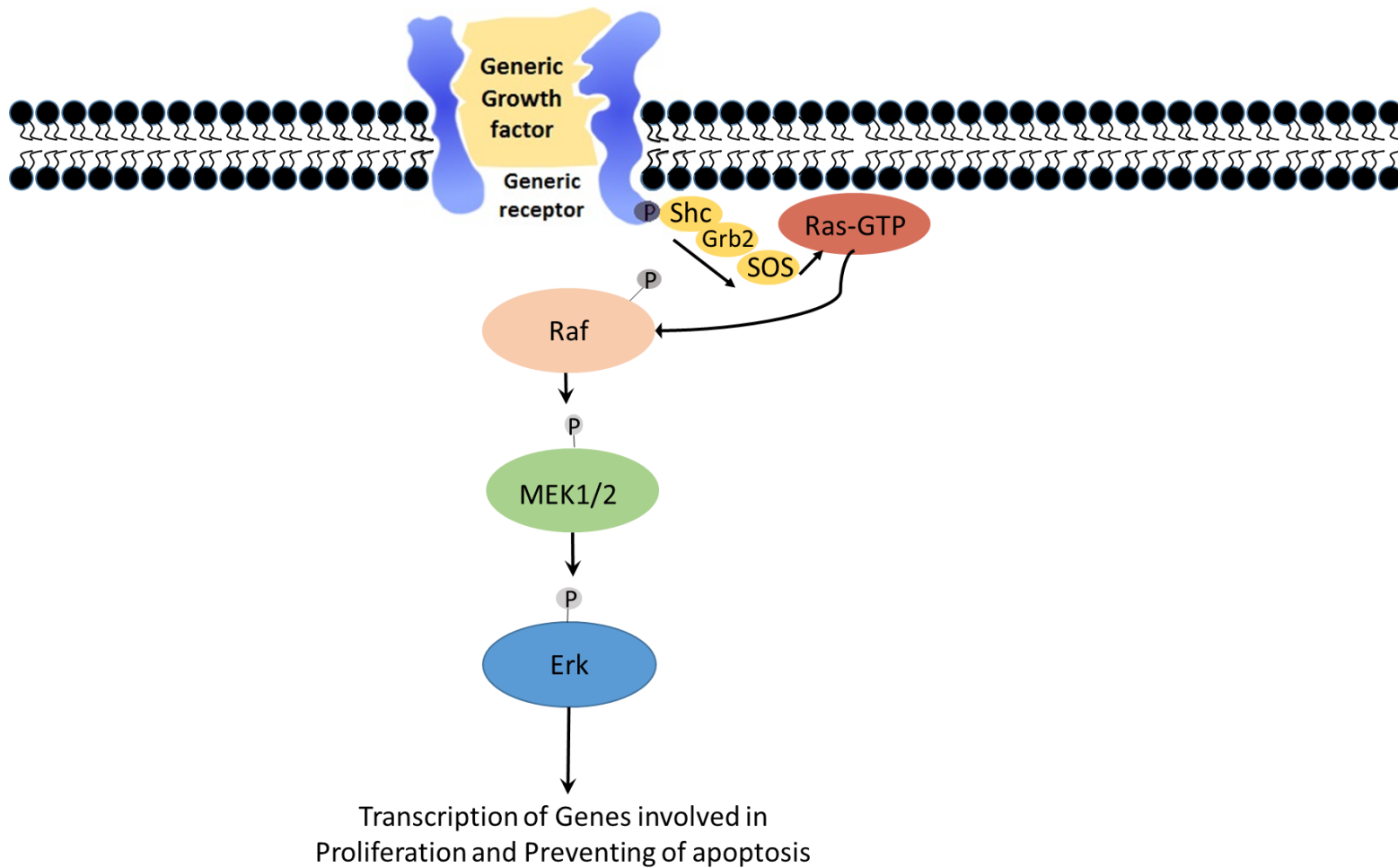
component of the rapid translocation of Erk to the nucleus. (Cobb and Goldsmith, 2000; Khokhlatchev et al., 1998) The phosphorylation of Erk results in a conformation change, which then results in the Dimer Interface of the protein being exposed, allowing two Erk molecules to form a dimer. (Canagarajah et al., 1997) The import of the active dimerised phosphorylated Erk to the nucleus predominately requires energy and cytosolic factors. (Ranganathan et al., 2006) This is in contrast to the singular non-phosphorylated inactive Erk, which does not require any energy or carrier proteins to enter the nucleus. (Ranganathan et al., 2006) MEK, as mentioned previously when inactive, will bind to Erk that has not been activated, this will keep it localised in the cytoplasm. (Fukuda et al., 1997) In addition MEK has been shown to be involved in the nuclear export of monomers of inactive Erk. (Adachi et al., 2000) The dimerisation of Erk has been suggested to hide the MEK binding site, further facilitating the localisation of Erk to the nucleus. (Cobb and Goldsmith, 2000)

In addition to MEK there are other proteins that interact with Erk to regulate its location throughout the cell. (Ramos, 2008) One other example is Sef, which can bind to Erk and blocks its nuclear localisation, but it does not prevent its phosphorylating activities in the cytoplasm. (Torii et al., 2004) It does this by binding to active MEK and preventing active Erk from dissociating from MEK. (Torii et al., 2004) The Sef/pMEK complex is located to the Golgi apparatus or the membrane ruffles, (Ramos, 2008) suggesting that Sef is involved in locating active Erk signalling to these parts of the cell. In contrast Mxi2 has been identified as a protein that binds directly to Erk, promoting its accumulation in the nucleus, (Casar et al., 2007) and prolonging its activation while having no effect on Erk's cytoplasmic function. (Sanz-Moreno et al., 2003)

Non phosphorylating inhibitors of Erk/MAPK signalling also exist. RKIP binds to Raf and MEK, inhibiting their ability to interact with each other (Yeung et al., 1999) and a loss of RKIP results in increased Erk signalling. (Ramos, 2008) Several metastatic cancers have been shown to downregulate RKIP (Ramos, 2008) and its over expression reduces the invasive potential of cancer cells. (Al-Mulla et al., 2013; Das et al., 2012; Schuierer et al., 2004) RKIP is believed to play an important role in suppressing metastasis. (Keller, 2004)

Therefore it is clear that regulation of the Raf/MEK/Erk signalling pathway is complex, and that specific signals are activated to regulate the intensity and duration of Erk signalling, depending on the requirements of the cell at that time. This is important because Erk has so many different possible targets that precise regulation of the pathway is required to ensure the correct targets are activated at the correct time. Loss of regulation (by K-Ras mutation for example) of this pathway can result in increasing the tumorigenic potential of a cell.





**Figure 1-3: Diagram of the Raf/MEK/Erk signalling pathway. Raf can be phosphorylated by Ras and other kinases. This phosphorylation of Raf activates it and promotes phosphorylation of its downstream targets, and ultimately the activation of multiple downstream genes.**

#### 1.4.2 PI3K MEDIATED SIGNALLING

The PI3K/Akt signalling pathway is also downstream of Ras and there are several areas of cross talk between the Raf/MEK/Erk and PI3K/Akt pathways. Mutation of Akt to express the dominant active form was shown not to be sufficient for PanIN formation in mice, but did lead to the formation of ductal structures and activation of progenitor cell associated genes. (Elghazi et al., 2009) Expression of the mutant form of Raf when TP53 is mutated was sufficient to cause the development of lethal PDACs, but the dominant active form of PI3K was not. (Collisson et al., 2012) However, PI3K has been shown to be required for the initiation and maintenance of carcinogenesis in the pancreas. (Eser et al., 2013) It would therefore appear that PI3K is not required to mimic the effects of K-Ras activation during PDAC formation in mice, but MAPK signalling is.

Once the tumour has formed, the requirements for signalling may be different to those required for the initiation of tumour formation. This was tested using pharmacological inhibitors. Inhibition of MEK with PD325901 causes tumour growth to be halted in KPC mice. Mice that were treated with this inhibitor survived longer than those without it, but they died shortly after the treatment was stopped. (Collins et al., 2012) Raf inhibitors by contrast did not prevent the growth of pancreatic cancer cell lines when they were treated and in fact interfered with the MEK inhibitors when the compounds were applied together. (di Magliano and Logsdon, 2013; Hofmann et al., 2012) The combined treatment of MEK inhibitors and Akt inhibitors in multiple different pancreatic cancer cell lines did however, have a synergistic anti-proliferative effect which was shown using several different inhibitors of each pathway in combination.

(Hofmann et al., 2012) Furthermore, the combination of these drugs in mice caused a regression in their tumours, but either drug alone only slowed the growth of the tumour. (Hofmann et al., 2012) It seems that both Akt and Erk signalling are important for the maintenance of tumour progression, and perhaps the cross talk between the two pathways can negate some of the effects of inhibiting earlier parts of the pathway.

### 1.4.3 RAL GTPASES

Ras-like GTPases (Rals) were initially discovered in a screen of simian B lymphocytes using a conserved 7 amino acid sequence from Ras, RhoA and yeast YPT Rab protein. (Neel et al., 2011) The Ral A gene was shown to have 50% sequence homology with genes of the Ras family. The sequence for simian Ral A was used to isolate the human homolog along with another gene *RALB*. (Chardin and Tavitian, 1986) Although these genes share similar sequence homology (82%) they have different functional roles within the cell. Rals are well conserved throughout species with *Caenorhabditis elegans* and *Drosophila melanogaster* encoding Ral GTPases, which are similar to RalA/B. (Neel et al., 2011)

Ral A and B require the binding of GTP to become active in a similar manner to Ras; as with Ras, GEFs are needed to promote GTP binding. The first RalGEF (RalGDS) was found in mice when looking for genes that shared a similar sequence to yeast RasGEFs. (Albright et al., 1993) RalGDS was found to have sequence homology with several of the active regions of RasGEFs, but they did not demonstrate activity with Ras. They were instead shown to be involved in the exchange of GTP for GDP in both Ral A and Ral B. (Neel et al., 2011) It appears that several of the Ral GEFs can also interact with Ras, and that Ras appears to be involved in activating them. (Ferro and Trabalzini,

2010) Ral therefore is another downstream effector of Ras. Ral is known to interact with several downstream effectors such as Ral binding protein 1 (RalBP1), which was the first effector discovered. (Cantor et al., 1995) Active RALs bind to the conserved Ral binding domain of RalBP1 and regulate its subcellular localisation, but not its activity or function. (Matsubara et al., 1997) RalBP1 has been demonstrated to contain two ATP binding sites and is involved in the transport of small molecules including anti-cancer drugs. (Vatsyayan et al., 2010) The inhibition of RalBP1 expression has been demonstrated to result in a regression in tumours of multiple different xenographs. (Neel et al., 2011)

In H460 (a K-Ras mutant lung cancer cell line) survivin was shown to be degraded when K-Ras or RalA was depleted. Survivin is a target of the Anaphase Promoting Complex/cyclosome (APC/c). Targeting RalA or K-Ras for depletion caused a greater decrease in survivin levels than when RalB was depleted. By contrast when another lung carcinoma cell line (A549) was tested it was shown to be more dependent on RalB to regulate the level of survivin. (Tecleab and Sebti, 2013) Depletion of RalB in this cell line was even more potent than K-Ras depletion. (Tecleab and Sebti, 2013) This degradation could not be replicated by the depletion of Raf-1 or Akt1/2, suggesting that the regulation of survivin levels in these lung cancer cell lines was mediated through the Ral pathway, rather than the more classically considered effectors of Ras signalling. (Tecleab and Sebti, 2013)

#### 1.4.4 MYC

Myc (gene MYC) is a transcription factor that is known to be involved in many different pathways that regulate growth, apoptosis, differentiation and proliferation of

cells. (Cole, 1986; Luscher and Eisenman, 1990; Prendergast, 1999) In pancreatic cancer the region of the chromosome containing the MYC gene has been shown to have an increased copy number, which has been suggested to be related to its overexpression in pancreatic cancer cells. (Schleger et al., 2002) Myc is activated by Ras/Raf MEK/Erk pathway through phosphorylation of serine 62 which stabilises the Myc protein. Phosphorylation of threonine 58 by GSK- $\beta$  results in Myc destabilisation, but this can only occur after Serine 62 has been phosphorylated. (Lutterbach and Hann, 1994; Sears et al., 2000) Ras via PI3K/Akt can inhibit GSK- $\beta$ , and therefore activation of Ras is able to stabilise Myc as well as prevent the destabilisation of the stable form by the inhibition of GSK- $\beta$  activity. (Sears, 2004) When active, Myc binds to the promoter regions of several key components of the cell cycle such as cyclins and several cyclin dependent kinases. (Cdks) (Bretones et al., 2014) It has therefore been determined to be involved in the cell cycle and provides a pathway by which Ras expression interacts with the cell cycle.

### 1.5 **PHOSPHOTIDYLCHOLINE AS A LIPID MEMBRANE COMPONENT.**

Phosphatidylcholine (PC) is a vital component of lipid membrane formation and cell signalling. In mammalian cells PC is synthesised from choline via the Kennedy (CDP-choline) pathway in most cell types, and is the main component of multiple phospholipids found in the membrane such as: phosphatidylinositol, (PI) phosphatidylserine, (PS) and phosphatidylethanolamine (PE) (Janardhan et al., 2006) (see Figure 1-4). PC is degraded via a specific phospholipase D (PLD) and both the creation and degradation are regulated to create PC homeostasis. Fluctuation of PC levels (either increased or decreased) can result in cell death. (Janardhan et al., 2006)

Stimulation by mitogenic growth factors or oncogenic transformation results in PLD mediated hydrolysis of PC into choline and phosphatidic acid (PA). This choline along with choline that has been acquired from outside sources enters the Kennedy pathway (Figure 1-4). (Hernandez-Alcoceba et al., 1999) Choline is then converted through several intermediates to PC, which can then be used as a building block for several membrane phospholipids. Phosphocholine (PCho) is one of the intermediates generated through the Kennedy pathway, and its level is controlled through the activity of Choline Kinase (ChoK) and PLD. (Janardhan et al., 2006) PCho is stable and essential in the induction of DNA synthesis (Cuadrado et al., 1993) and so PCho, via the Kennedy pathway is thought to be an important step in the regulation of cell proliferation and malignant transformation. (Rodriguez-Gonzalez et al., 2003)

Mutant Ras can enhance the activity of ChoK through the Ral-GDS and PI3K pathways. (Ramirez de Molina et al., 2002) The inhibition of ChoK appears to be able to be an effective antitumor strategy against cells transformed in this manner. (Ramirez de Molina et al., 2001) Raf signalling was shown not to have a significant effect on ChoK activity. (Ramirez de Molina et al., 2002) It has therefore been demonstrated that mutant Ras can affect ChoK activity. Ras may therefore be able to alter the lipid profiles of the membranes of transformed cells. In support of this the genesis of lymphoma by activation of different oncogenes (Myc and Ras) results in a change in lipid profile. (Eberlin et al., 2014)

## 1.6 **K-RAS AS A TARGET FOR TREATMENT**

The global presence of Ras isoforms in cellular signalling, coupled with the high frequency of K-Ras mutations, make it an attractive target for clinical therapies. At the time of writing, a successful treatment has not been developed.(Macdonald et al., 2005) It may be that no effective small molecule has been identified, but it may also be due to the ability of subpopulations of pancreatic cancer cells to survive the loss of K-Ras. The surviving cell (SC) population may be able to aggressively repopulate following the end of treatment. (Viale et al., 2014) There may even be a population that could survive with continued treatment because they have become independent of K-Ras. (Singh et al., 2009)

A potential new treatment featuring compounds that bind specifically to p.G12C K-Ras mutations has been demonstrated in cell lines. Binding of these compounds inhibits the function of mutant K-Ras in two ways. It increases the affinity of the mutant K-Ras to GDP instead of GTP resulting in its inactivation, and it also diminishes the interaction that K-Ras can have with its downstream targets. (Ostrem et al., 2013) The specificity of this treatment means that it does not affect wild type K-Ras. However, this is not an effective treatment for other K-Ras mutants and so identification of other agents is required. Characterisation of pathways downstream of K-Ras (perhaps undiscovered so far) that are involved in cellular processes such as growth and maintenance of the cancer cell, will offer potential alternative targets for treatment that can be used independently or in combination with K-Ras inhibition.

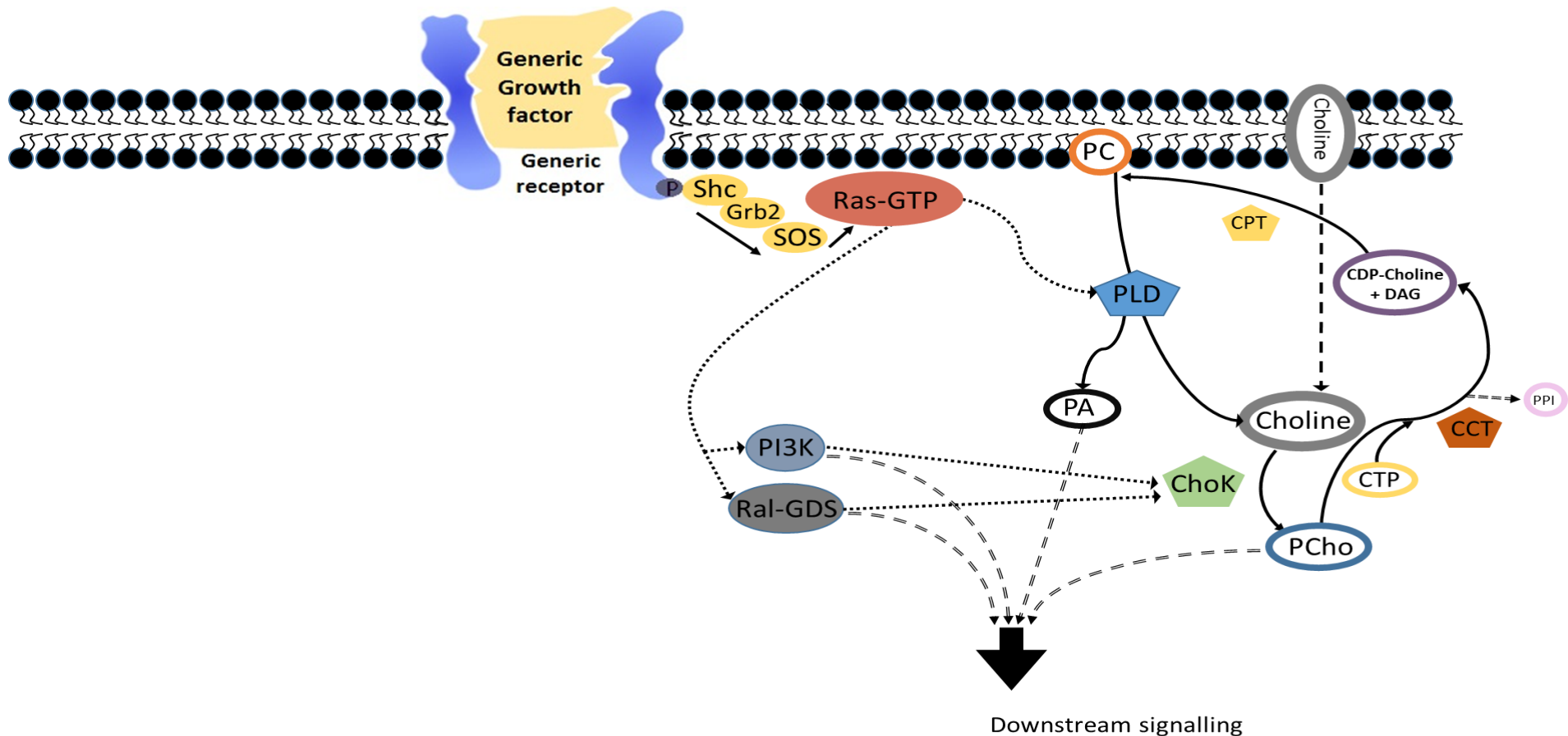


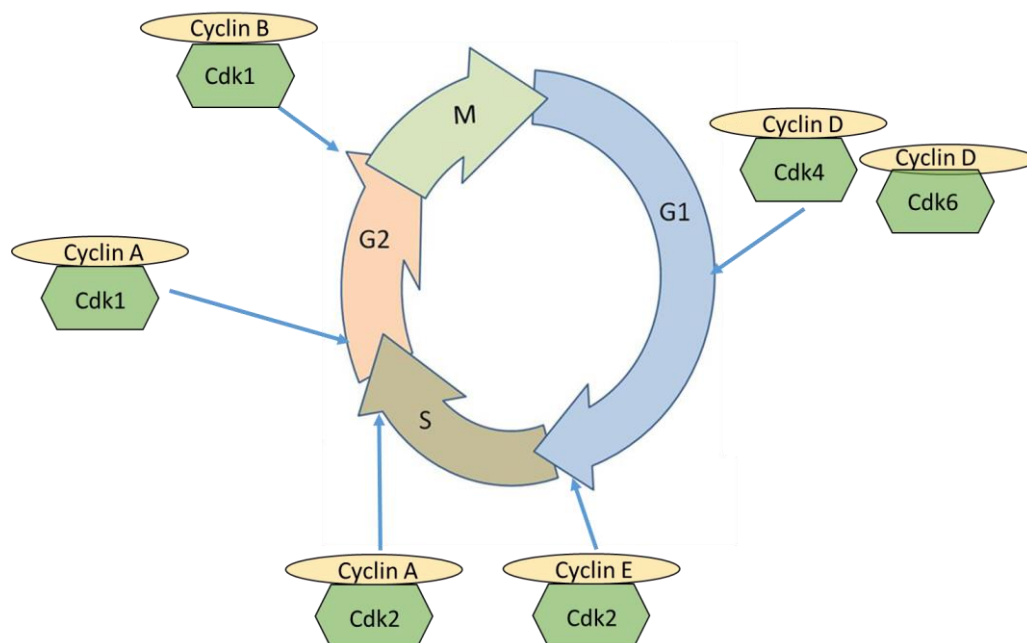
Figure 1-4: Diagram showing the synthesis of Phosphatidylcholine from PCho via the CDP-choline Kennedy pathway. Ras can modulate the Kennedy pathway and therefore regulate the levels of different components of the pathway. This regulation allows Ras to control the makeup of the lipid membrane, as well as altering signalling downstream.



## 1.7 CELL CYCLE

### 1.7.1 OVERVIEW

The cell cycle can be thought of as two separate parts: mitosis (M) where nuclear division occurs and interphase, which is the phase between two concurrent mitoses. (Figure 1-5). Mitosis is divided into prophase, metaphase, anaphase, telophase, and cytokinesis. There are three separate parts to interphase (G1, S and G2 phases) (Figure 1-5) . (Norbury and Nurse, 1992) Cells grow in size during G1 (hence the archaic name of ‘Growth’ phase) before entering S phase to replicate their DNA. Following S phase the cell enters G2 where it prepares for cell division that will occur in mitosis. Cells can also move into G0 from G1 where they are in a non dividing state. This quiescent state is where most normal cells reside.(Vermeulen et al., 2003)



**Figure 1-5: Diagram demonstrating the phases of the cell cycle. The main cyclins and Cdks are shown with their most recognised point of action demonstrated, other cyclin-Cdk interactions (although important) are not included for simplicity.**

### 1.7.2 CONTROL OF THE CELL CYCLE

As mentioned previously, the cell cycle is divided into several different phases that have to be completed sequentially to successfully result in the genesis of two daughter cells. Cyclin dependent kinase (Cdks) are responsible for the regulation of gene expression at the different stages of the cell cycle (Figure 1-5). This control can occur through the activation of transcription factors that promote the transcription of the genes of interest. Cyclin E/Cdk2 for example, can phosphorylate pRB (Retinoblastoma protein) which is inhibitory when bound to the transcription factor E2F. pRB phosphorylation dissociates it from E2F increasing its ability to activate transcription (Figure 1-6). (Dyson, 1998; Takahashi et al., 2000) Different Cdks are found to be active in different parts of the cell cycle, but the levels of Cdks tend to be constant. The levels of Cdk activator proteins (cyclins) oscillate throughout the cell cycle and dictate the Cdks that are active and when. (Morgan, 2007) Activation of Cdks has to occur in a sequential manner and once it starts the cell will complete a full cycle. This is to prevent multiple incomplete firings of the cell cycle which could lead to mistakes being made in cell division and usually cell death.(Morgan, 2007)

Cdks have a similar structure to other protein kinases with two lobes forming a cleft into which ATP fits. (De Bondt et al., 1993) There are two additional modifications compared to other protein kinases. The modifications have been identified by studying the crystal structure of human Cdk2. They are firstly a large loop structure that blocks entry into the substrate binding pocket. Secondly, several of the amino acid side chains are not located in the standard position within the ATP binding site. (De Bondt et al., 1993) Both of these features keep Cdks inactive when they are not bound to cyclins.

Cyclins are different from each other in their amino acid sequences. However, they all contain a conserved 100 amino acid sequence known as the cyclin box.(Brown et al., 1995; Petri et al., 2007) Despite the differences in sequence, their tertiary structures always contains a region known as the cyclin fold. This region consists of two domains of 5  $\alpha$ -helices. One of these domains is made up of the cyclin box, the other is made up of a similar arrangement of helices although the primary structures can contain little homology. (Brown et al., 1995; Petri et al., 2007) This cyclin fold region is also found in several transcriptional regulators, which may indicate that they have evolved from a common ancestor protein. (Morgan, 2007)

In addition to the binding of cyclins to the Cdk, further phosphorylation at a threonine residue next to the active site is required for full activation. (Morgan, 1997) Cdk-activating kinases (CAKs) are responsible for the phosphorylation of this residue to complete the activation of Cdks. (Harper and Elledge, 1998; Morgan, 2007) Unlike most phosphorylation modifications this one appears to be irreversible but it only occurs in mammalian cells after the binding of cyclins. (Morgan, 2007) The CAKs appear to be at high levels throughout the cell cycle, therefore, it is the binding of cyclins that is the rate limiting step for the activation of Cdks. (Morgan, 2007) In addition to these activating mechanisms, phosphorylation of Tyrosine 15 and Threonine 14 of Cdks is inhibitory. Both of these residues are found in the roof of the ATP binding pocket and their phosphorylation likely interferes with the orientation of the ATP phosphates. Thus preventing the Cdks ability to perform its kinase function. The phosphorylation state of these residues is closely regulated by kinases and phosphatases. In eukaryotes, the dephosphorylation is mediated by members of the Cdc25 family and phosphorylation is carried about by Wee1 (Tyr 15) and Myt1 (Tyr 14 and 15). (Dunphy, 1994)

The crystal structure showing the conformational changes that occur when cyclins bind to Cdks, has been done initially using Cdk2 binding to cyclin A. It has been inferred to be similar for other cyclin/Cdk interactions. (Brown et al., 1999; De Bondt et al., 1993; Petri et al., 2007) The binding of different cyclins to the same Cdk demonstrates differing kinase activity towards the same target sequence. This suggests that some differences must exist in how the cyclins promote the kinase activity. (Morgan, 2007) Cyclin A binding interacts with both lobes of Cdk and the T-loop is moved from blocking the protein binding site. Additionally residues that are misaligned in the ATP binding pocket are now correctly aligned to allow the phosphotransfer reaction to take place. (Jeffrey et al., 1995; Russo et al., 1996) The binding of cyclin A results in conformational changes to Cdk2, allowing its substrate to bind as well as reconfiguring the ATP binding pocket to an active form. In addition, the binding of Cyclin A also provides a ridged framework, which supports Cdk2. (Jeffrey et al., 1995)

In addition to activating Cdks some cyclins can specifically target Cdk substrates. This potentially could be important in ensuring that the correct downstream targets are activated at the same time, because different cyclins can target the same Cdk at different points in the cell cycle. (Miller and Cross, 2001; Roberts, 1999) An example of this is that Cdk1 and Cdk2 will bind to p107 when they are bound by cyclin A, but not when bound by cyclin B. (Morgan, 2007; Peeper et al., 1993) This mechanism provides greater flexibility for regulating downstream targets than if specificity was defined by the Cdk alone. In addition cyclins are able to locate the cyclin/Cdk complex to specific parts of the cell, at various points in the cell cycle to regulate their function by partition. (Pines and Hunter, 1991)

Degradation of proteins via the proteasome (proteolysis) is an important mechanism of regulation for the cell cycle. It helps ensure that the cell cycle is sequential by degrading regulatory proteins when they are not required. Additionally, degradation of regulatory proteins after cell division has occurred can ensure that another round of cell division does not occur too quickly. Proteins that are designated to undergo proteolysis are tagged by multiple copies of the small protein ubiquitin tag in a multi-step process. (Pickart, 2001) Proteins that are tagged by ubiquitin are recognized by the proteasome and undergo proteolysis.

Of particular relevance to the cell cycle there are two different large multi-subunit ubiquitin-protein ligases that are responsible for correctly tagging proteins to be destroyed. SCF (its name derived from its key components Skp1, cullin and the F-box) is the key ligase complex for the ubiquitination of Cdks at the G1/S transition. (Orlicky et al., 2003; Zheng et al., 2002) The F-box of SCF determines the target specificity of the complex. With several different F-boxes being interchangeable to create specific targeting for different subsets of proteins. For example the F-box protein Cdc4 targets cyclin E for degradation. (Jin et al., 2004)

The other main complex that is important in the regulation of the degradation of cell cycle proteins is the Anaphase Promoting complex (APC/c). The APC/c is controlled by activator subunits binding which bind to the APC/c core proteins at different times during the cell cycle. Cdc20 and Cdh1 are two of these activators and they play a key role in the control of the cell cycle. Cdc20 is involved in activation of the APC/c when cells are transitioning from anaphase to metaphase, and promotes the segregation of sister chromatids and exit from mitosis.

Phosphorylation of core APC/c subunits by Cdk<sub>s</sub> increases the binding affinity for Cdc20. (Harper et al., 2002; Peters, 2002) Once bound APC<sup>Cdc20</sup> targets these same Cdk<sub>s</sub> for destruction and therefore generates a negative feedback loop on its activation. This destruction of Cdk<sub>s</sub> via APC<sup>Cdc20</sup> is a delayed event after activation, ensuring that it does not occur too early in mitosis. (Morgan, 2007; Peters, 2002) The destruction of Cdk<sub>s</sub> and their activating cyclins (A+B) results in less phosphorylation of the APC/c, therefore Cdc20 dissociates. Loss of Cdc20 binding to the APC/c results in its inactivation. This mechanism results in an inactive APC/c by the end of mitosis. (Harper et al., 2002; Peters, 2002)

All cells apart from early embryonic cells do not undergo another round of cell division immediately and these cells will enter G1. The reactivation of the G2 Cdk<sub>s</sub> and G2 cyclins would therefore be disruptive to this plan. To maintain the cells in G1, the G2 Cdk<sub>s</sub> and cyclins activity are inhibited, their transcription downregulated, and they are also degraded via the proteasome. These are targeted for degradation via the APC/c, however in G1 the APC/c is activated by the binding of Cdh1. (Harper et al., 2002; Peters, 2002) Cdh1 does not bind to the APC/c when it is phosphorylated by Cdk<sub>s</sub>. Therefore Cdh1 binds and regulates activity after the completion of mitosis when Cdc20 has dissociated, and the Cdk<sub>s</sub> that can phosphorylate the APC/c, are inactive. APC<sup>Cdh1</sup> therefore is active through G1, keeping cyclin B and its related Cdk<sub>s</sub> at low levels until the cell is ready to undergo another round of cell division. (Harper et al., 2002; Morgan, 2007; Peters, 2002) The G1 cyclins (cyclin D and E) are not recognized by APC<sup>Cdh1</sup> and therefore the activity of the G1/S Cdk<sub>s</sub> will rise as the cell approaches another round of cell division. (Harper et al., 2002) When the levels of the G1/S Cdk<sub>s</sub> have risen they

phosphorylate Cdh1, dissociating it from the APC/c, thereby inactivating it and allowing cyclin B levels to rise again. (Harper et al., 2002)

As previously discussed Cdk levels tend to be relatively stable (Sullivan and Morgan, 2007) and their activation is regulated by other proteins. The combination of these regulatory mechanisms results in a mechanism that can control complete and accurate formation of daughter cells. Cyclin D has 3 different isoforms (D1, D2, D3) with D1 being the most commonly considered. All three D type cyclins are able to bind to Cdk4 and Cdk6 (Figure 1-5). The binding and activation of these Cdks is required to allow the cells to enter G1 from G0. (Sherr, 1994) Once active Cdks will phosphorylate downstream targets (Morgan, 1995) and drive the cell cycle towards G2. (Sullivan and Morgan, 2007) Cyclin D is unique amongst cyclins because it is not expressed periodically, but will be expressed as long as it is stimulated by growth factors and is rapidly degraded following their loss. (Assoian and Zhu, 1997; Sherr, 1994) The loss of cyclin D during G1, results in cells not being able to enter S-phase but if cyclin D is lost later in the cell cycle, it does not appear to have any effect. (Baldin et al., 1993; Quelle et al., 1993; Sherr, 1994) The activation of cyclin D leads to the phosphorylation of RB. E2F/RB is a transcription repressor and phosphorylation of pRB (Retinoblastoma protein) by Cdk4/Cyclin D causes a reduction in this transcriptional repression. This leads to expression of a number of proteins including CDC6/hCdc18 (which is essential for maturation of the Origin Recognition Complex) and Cyclin E. (Dyson, 1998; Weinberg, 1995) Cyclin E activates Cdk2, which as a consequence, further phosphorylates pRB resulting in its dissociation from E2F. The RB free E2F “activator” protein binds to the promoter regions of CDC6/hCdc18 and cyclins A and E activating their transcription. (Sherr and Roberts, 1999; Takahashi et al., 2000) (Figure 1-6) Cyclin

A also has been demonstrated to be able to phosphorylate pRb. It is also believed that this activity maintains the phosphorylation as the cells pass through S-phase. (Classon and Harlow, 2002; Morgan, 2007) The increase in transcription of cyclins A and E along with the inactivation of the APC<sup>Cdh1</sup> (which degrades cyclin A) allows for the concentration of G2 cyclins to begin to increase as the cell passes through S-phase into G2. (Harper et al., 2002)

The loss of APC<sup>Cdh1</sup> allows the buildup of cyclin B/Cdk1. (Harper et al., 2002) However, its activity may be kept at a low level due to phosphorylation by Wee1 and Myt1 (as previously described). (Dunphy, 1994) As the cell progresses in G2 these complexes are dephosphorylated by Cdc25 (A-C), activating them. (Dunphy, 1994) This gives a positive feedback due to active cyclin B-Cdk1 complexes phosphorylating Cdc25 and the inhibitor kinases Wee1 and Myt1, activating and deactivating them respectively. (Domingo-Sananes et al., 2011) This results in a switch like response where cyclin B is generally either active or inactive (i.e. not graduated response); the caveat being partial activation of cyclin B-Cdk1 is required before the feedback loops are able to elicit a complete response. (Hoffmann et al., 1993) There is evidence demonstrating that cyclin A-Cdk2 complexes are able to phosphorylate Cdc25 (activating it) as well as both Myt1 and Wee1, resulting in their inactivation. (De Boer et al., 2008) Proteins of the 14-3-3 group (apart from 14-3-3 $\sigma$ ) have also been shown to be able to bind to Cdc25B/C that have been phosphorylated at a single binding site. The binding of 14-3-3 blocks Cdc25 interaction with cyclin B-Cdk1 and results in Cdc25 being sequestered in the cytoplasm. (Gardino and Yaffe, 2011) 14-3-3 $\sigma$  and other 14-3-3 isoforms are also able to bind Wee1 causing it to be evenly distributed in the nucleus. (Gardino and Yaffe, 2011)



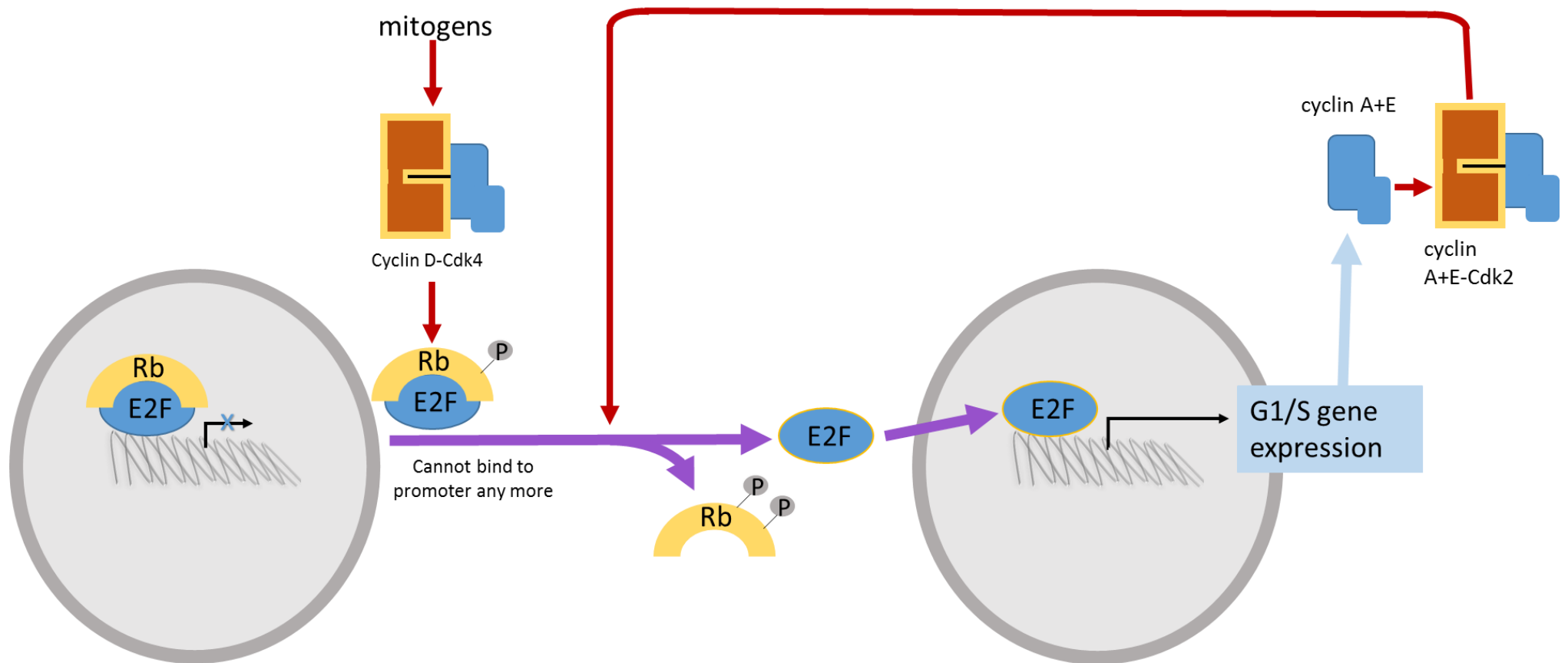


Figure 1-6: **Diagram showing how multiple phosphorylation events are required to fully activate cyclin A and E transcription.** E2F-Rb acts as a transcriptional repressor unless Rb is phosphorylated. Further phosphorylation causes loss of Rb and E2F can act as a transcriptional activator.

The Chk proteins are thought to be important in the cells response to DNA damage; with Chk1 being predominately responsible for response to single strand breaks and Chk2 being predominately involved in response to double strand breaks. (Jazayeri et al., 2006) Upon their activation, the Chk1/2 proteins have been shown to inactivate Cdc25A and increase the activation of Wee1, therefore preventing cells with DNA damage entering mitosis until the damage has been repaired. (Reinhardt and Yaffe, 2009)

AMP-activated Protein Kinase (AMPK) directly inhibits Protein Phosphatase 1 Regulatory Subunit 12C (PPP1R12C) by phosphorylation. (Banko et al., 2011) PPP1R12C is known to inhibit the function of the myosin regulatory light chain (MRLC) by removing activating phosphates. (Banko et al., 2011; Ito et al., 2004; Komatsu et al., 2000) The loss of this activation prevents cytokinesis (Banko et al., 2011; Komatsu et al., 2000) and therefore a lack of proper cell division, resulting in an increase in the number of multinucleated cells.

The active form of AMPK (pAMPK) has a role in preventing the cell passing a 'lipid' checkpoint in mitosis. (Scaglia et al., 2014) Scaglia *et al*, provide evidence that the lipid checkpoint ensures the synthesis of fatty acids and phosphatidylcholine is available for the generation of new membranes. They show that pAMPK activity decreases the levels of Acetyl-CoA carboxylase 1 which is the rate-limiting enzyme in fatty acid synthesis. (Scaglia et al., 2014) The presence of pAMPK therefore results in the loss of de novo fatty acid synthesis and subsequently cell arrest at this point.

In addition to its activation, relocation of cyclin-B-Cdk1 to the nucleus is important for its functionality in the cell. (Pines and Hunter, 1991) In G2 and early prophase, the complex tends to be located in the cytoplasm before it enters the nucleus, triggering the

nuclear envelope to dissolve and allowing cyclin B-Cdk1 to affect nuclear targets at the correct time. (Morgan, 2007; Pines and Hunter, 1991)

After the separation of chromatids, the Cdk targets that have been activated through phosphorylation have to be deactivated, this is the major regulatory mechanism for the completion of mitosis. Most of this deactivation occurs by deactivation of the Cdks so that phosphatases can remove the phosphates from the activated Cdk targets. This degradation of cyclin B is achieved using the APC<sup>Cdc20</sup> as previously described. (Harper et al., 2002; Peters, 2002) The APC<sup>Cdc20</sup> is not enough to inactivate the genes activated by Cdk1 complexes, Cdc14 dephosphorylates cyclin B-Cdk1 and its targets, inactivating them. (Jaspersen et al., 1998) This results in decreased kinase activity of the Cdks, which turns off the downstream targets and eventually results in Cdc20 dissociating from the APC/c (as its phosphorylation by Cdks fails to occur), to be replaced by Cdh1. Cdh1 binding to the APC/c completes the inactivation of Cdk1, returning the cell to G1 to await another round of cell division. (Harper et al., 2002; Morgan, 2007; Peters, 2002)

### 1.8 MYC INVOLVEMENT IN THE CELL CYCLE

Myc is a transcription factor that binds to the promotor region of several different genes whose products are vital for cell cycle control and progression (Bretones et al., 2014) and is a known downstream target of Ras (previously described). It is able to help promote the G1/S transition by promoting the transcription of the cyclins and Cdks. Additionally it is also able to induce CAKs and Cdc25 proteins and inhibit Wee1, (Bretones et al., 2014) resulting in greater activation of the various cyclin Cdk complexes (e.g. cyclin B/Cdk1) that, as previously described, are required to allow the

cells to transition from G1 to S-phase. Myc therefore provides one way for the mutation of K-Ras in pancreatic cancer cells to influence a key ‘Hallmark of Cancer’, cell proliferation. (Figure 1-34). (Hanahan and Weinberg, 2000, 2011)

## 1.9 AIMS

K-Ras is well known to be involved in pancreatic cancer and has several well studied functions. There is little previous evidence regarding the contribution of other Ras isoforms to the development and maintenance of PDAC. Additionally, although K-Ras has been identified as controlling cyclin D, there has been little research on its role regulating the other cyclins. The aims of this project and my initial hypotheses are therefore:

- To identify if Ras isoforms other than K-Ras, are active in pancreatic cancer cells, and if they have any functions that differ to K-Ras.
  - *Hypothesis: no other Ras isoforms are active or functional in pancreatic cancer cells.*
- To examine how active Ras isoforms modulate cyclin levels in PDAC cell lines and identify if cyclin regulation extends beyond the classical paradigm described for cyclin D.
  - *Hypothesis: K-Ras only regulates the G2 cyclins via cyclin D in a cell cycle dependent manner.*

## CHAPTER 2: MATERIALS AND METHODS

### Cell lines

Cell line	Where obtained
Suit-2	Gift from Professor Nick Lemoine, Barts Cancer Institute, Queen Mary University of London
Panc-1 slow	American Type Culture Collection (Middlesex, UK)
Panc-1	American Type Culture Collection (Middlesex, UK)
BxPC3	American Type Culture Collection (Middlesex, UK)
MIA PaCa-2	American Type Culture Collection (Middlesex, UK)
KPC mouse derived cell line	Gift from Michael C Schmid University of Liverpool

## Suppliers of chemicals and reagents

<b>Chemical or Reagent</b>	<b>Supplier</b>	<b>Catalogue number</b>
6-well tissue culture plates	Appleton Woods, Birmingham	CC010
96-well tissue culture plates	Appleton Woods, Birmingham	BC015
Albumin from Bovine Serum	Sigma-Aldrich Company Ltd, Gillingham, Dorset	A4503-100G
Amphotericin B solution	Sigma-Aldrich Company Ltd, Gillingham, Dorset	A2942-20ML
Monoclonal Anti- $\beta$ -Actin antibody produced in mouse	Sigma Chemical Co, Poole, Dorset	A5441-.2ML
Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay	Bio-Rad Laboratories Ltd, Hercules, CA, USA	M50-0KCAF0Y
Cyclin D1 antibody	Abcam, Cambridge, Uk	ab137875
Cyclin E antibody	Santa Cruz Biotechnology, Heidelberg, Germany	sc-198
Cyclin A antibody (H-432)	Santa Cruz Biotechnology, Heidelberg, Germany	sc-751
Cyclin B antibody	Santa Cruz Biotechnology, Heidelberg, Germany	sc-752
N-Ras antibody	Santa Cruz Biotechnology, Heidelberg, Germany	sc-31
geminin (F-7) antibody	Santa Cruz Biotechnology, Heidelberg, Germany	sc-74456
survivin antibody (D-8):	Santa Cruz Biotechnology, Heidelberg, Germany	sc-17779
Anti-phospho-AMPK $\alpha$ (Thr172) antibody	Merck Millipore, Watford, UK	07-681
Pierce Active Ras pulldown and detection Kit	Fisher Scientific, Loughborough, Leicestershire	11825015

Bortezomib	Santa Cruz Biotechnology, Heidelberg, Germany	sc-217785
Protein assay reagent A, BCA	Fisher Scientific, Loughborough, Leicestershire	11881345
Protein assay reagent B, BCA	Fisher Scientific, Loughborough, Leicestershire	10475944
Cell scrapers	STARLAB (UK) Ltd, Milton	CC7600-0250
15 ml Centrifuge tube, conical, loose (Sterile)	STARLAB (UK) Ltd, Milton Keynes, Buckinghamshire	E1415-0200
50 ml Centrifuge tube, conical, loose (Sterile)	STARLAB (UK) Ltd, Milton Keynes, Buckinghamshire	E1450-0200
Click-iT® EdU Alexa Fluor® 647 flow cytometry assay kit	Life technologies, Paisley, Renfrewshire	C10424
Dimethyl sulfoxide (DMSO)	Fisher Scientific, Loughborough, Leicestershire	D8418-1L
Distilled water	University of Liverpool stores	
DL-dithiothreitol (DTT)	Sigma-Aldrich Company Ltd, Gillingham, Dorset	D0632-5G
Dulbecco's Modified Eagle's Medium (DMEM)	Life technologies, Paisley, Renfrewshire	31966047
Ethanol	University of Liverpool stores	
0.5 ml Eppendorf (SafeLock) microcentrifuge Tube	Fisher Scientific, Loughborough, Leicestershire	10292611
1.5 ml Eppendorf (SafeLock) microcentrifuge Tube	Fisher Scientific, Loughborough, Leicestershire	10509691
FACS tubes BD falcon round bottom polystyrene tube 5 ml with Cap	Scientific Laboratory Supplies Limited, Wilford, Nottingham	352054
Foetal bovine serum (FBS) (South American)	Life technologies, Paisley, Renfrewshire	10270106
GDC-0941 (PI3K inhibitor)	Stratech Scientific Ltd, Newmarket, Suffolk	S1036-SEL-5mg
Glycerol	Fisher Scientific, Loughborough, Leicestershire	10579570



Glycine	Fisher Scientific, Loughborough, Leicestershire	10607443
Goat anti-mouse immunoglobulin HRP	Dako Ltd, High Wycombe, Buckinghamshire	DAKO P0447
Goat anti-rabbit immunoglobulin HRP	Dako Ltd, High Wycombe, Buckinghamshire	DAKO P0448
Hs_CCNA2_1_SG QuantiTect primer Assay	Qiagen Ltd, Manchester, UK	QT00014798
Hs_CCNB1_1_SG QuantiTect primer Assay	Qiagen Ltd, Manchester, UK	QT00006615
Hs_GAPDH_1_SG QuantiTect primer Assay	Qiagen Ltd, Manchester, UK	QT00079247
Hydrochloric acid	University of Liverpool stores	
Carestream® Kodak® autoradiography GBX developer	Sigma-Aldrich Company Ltd, Gillingham, Dorset	P7042-5GA
Carestream® Kodak® autoradiography GBX fixer/replenisher	Sigma-Aldrich Company Ltd, Gillingham, Dorset	P7167-5GA
L-Glutamine solution Biotra, 200 MM	Sigma-Aldrich Company Ltd, Gillingham, Dorset	G7513-100ML
LightCycler 480 Sybr Green master mix	Roche Diagnostics Ltd, West Sussex	4707494001
LightCycler 480 plates 96 well	Roche Diagnostics Ltd, West Sussex	4729692001
Lipofectamine (15 ml)	Life technologies, Paisley, Renfrewshire	11668500
Mini Protean® Tetra System/AnyKD gels	BioRad, Hemel Hempstead, Hertfordshire	456-9033
Mini Protean® Tetra System/ TURBO PVDF MINI, 10	BioRad, Hemel Hempstead, Hertfordshire	170-4156
Non-fat Dry Milk – blotting grade	BioRad, Hemel Hempstead, Hertfordshire	170-6404
Nucleocounter solution A	ChemoMetec A/S Allerod, Denmark	910-0003
Nucleocounter solution B	ChemoMetec A/S Allerod,	910-0002

	Denmark	
Nucleocounter cassettes	ChemoMetec A/S Allerød, Denmark	940-0002
Nunc flask T25 vented	Fisher Scientific, Loughborough, Leicestershire	12034917
Nunc flask T75 vented	Fisher Scientific, Loughborough, Leicestershire	10364131
Nunc flask T175 vented	Fisher Scientific, Loughborough, Leicestershire	10246131
Opti-MEM® I	Life technologies, Paisley, Renfrewshire	31985-062
PD0325901 (MEK inhibitor)	Stratech Scientific Ltd, Newmarket, Suffolk	S1036-SEL-5mg
Penicillin-streptomycin	Sigma-Aldrich Company Ltd, Gillingham, Dorset	P0781-100ML
Phosphate Buffered Saline (PBS) Tablets	Fisher Scientific, Loughborough, Leicestershire	10388739
Phosphate Buffered Saline (PBS) (Sterile used for cell culture)	Sigma-Aldrich Company Ltd, Gillingham, Dorset	20012019
10x Phosphate Buffered Saline (PBS) (non sterile)	Fisher Scientific, Loughborough, Leicestershire	10214733
Corning 5 ml pipettes	Appleton Woods, Birmingham	CC208
Corning 10 ml pipettes	Appleton Woods, Birmingham	CC214
Corning 25 ml pipettes	Appleton Woods, Birmingham	CC216
10 µl graduated tip, refill	Starlab, Milton Keynes, Buckinghamshire	S1111-3700
200 µl yellow bevelled tip, refill	Starlab, Milton Keynes, Buckinghamshire	S1111-1706
1000 µl blue graduated tip, refill	Starlab, Milton Keynes, Buckinghamshire	S1111-6701
1-200ul round gel loading tip, racked	Starlab, Milton Keynes,	

(sterile)	Buckinghamshire	I1022-0610
10 µl XL graduated filter tip (sterile)	Starlab, Milton Keynes, Buckinghamshire	S1120-3810
20 µl bevelled filter tip (Sterile)	Starlab, Milton Keynes, Buckinghamshire	S1120-1810
200 µl graduated filter tip (Sterile)	Starlab, Milton Keynes, Buckinghamshire	S1120-8810
1000 µl XL filter tip (Sterile)	Starlab, Milton Keynes, Buckinghamshire	S1122-1830
Pre-diluted protein assay Standard (BSA V) Set	Sigma-Aldrich Company Ltd, Gillingham, Dorset	1053-1674
Propidium Iodide	Sigma Chemical Co, Poole, Dorset	P4170-10MG
Protease inhibitor tablets, EDTA-free	Roche, Welwyn Garden City, Hertfordshire	11422900
Protein ladder	Life technologies, Paisley, Renfrewshire	26617
Reagent reservoirs	STARLAB (UK) Ltd, Milton	E2310-1010
QuantiTech reverse transcription kit	Qiagen Ltd, Manchester, UK	205311
RNase A/T1 Mix	Fisher Scientific, Loughborough, Leicestershire	EN0551
RNeasy Mini Kit	Qiagen Ltd, Manchester, UK	74104
RPMI-1640 media	Sigma Chemical Co, Poole, Dorset	R0883- 6X500ML
RPMI-1640 media 10x bicarbonate free	Sigma Chemical Co, Poole, Dorset	R1145-500ML
SMARTpool: ON-TARGETplus CCND1 siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	L-003210-00- 0010
SMARTpool: ON-TARGETplus RALA siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	L-009235-00- 0005
SMARTpool: ON-TARGETplus RALB siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	L-008403-00- 0005
SMARTpool: ON-TARGETplus N- Ras siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	L-003919-00- 0010

SMARTpool: ON-TARGETplus K-Ras siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	L005069-00-0020
ON-TARGETplus KRAS ORF siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	J-005069-10-0050
ON-TARGETplus KRAS 3'-UTR siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	J-005069-11-0050
ON-TARGETplus KRAS ORF siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	J-005069-08-0050
ON-TARGETplus KRAS UTR, ORF siRNA	GE healthcare/Dharmacon Lafayette, CO, USA	J-005069-09-0050
ON-TARGETplus Non-targeting Pool	GE healthcare/Dharmacon Lafayette, CO, USA	D-001810-10-05
ON-TARGETplus RISC free control	GE healthcare/Dharmacon Lafayette, CO, USA	D-001600-01-05
Sodium chloride	Fisher Scientific, Loughborough, Leicestershire	10428420
Sodium dodecyl sulphate (SDS)	Fisher Scientific, Loughborough, Leicestershire	10607443
Sodium hydroxide	VWR, Lutterworth, Leicestershire	10396240
Supreno, blue, powder-free nitrile gloves	Starlab, Milton Keynes, Buckinghamshire	SU-INT-M
Trypsin-EDTA solution	Sigma Chemical Co, Poole, Dorset	T3924-100ML
TC10 cell counting slides	BioRad, Hemel Hempstead, Hertfordshire	145-0011
Tris base	Fisher Scientific, Loughborough, Leicestershire	10376743
Triton X-100	Sigma-Aldrich Company Ltd, Gillingham, Dorset	X100-100ML
Tween® 20	Sigma-Aldrich Company Ltd, Gillingham, Dorset	P1379-500ML
Ubiquitin enrichment kit	Fisher Scientific, Loughborough, Leicestershire	10158023
Virkon	Fisher Scientific,	HYG-205-021K

	Loughborough, Leicestershire	
Western lightning plus ECL	Life technologies, Paisley, Renfrewshire	31985-062
X100 FUJI RX X-Ray film	Fisher Scientific, Loughborough, Leicestershire	1271-5325
XF24 flux pak (seahorse kit)	Seahorse BioSciences order online at seahorsebiostore.com	100850-001

### Suppliers of equipment

Branson sonicade	Branson Ultrasonics Co, Shangai, China	
Bio-Plex 200 System	Bio-Rad Labororatories Ltd, Hercules, CA, USA	
Bio-Plex Pro wash station	Bio-Rad Labororatories Ltd, Hercules, CA, USA	
Bio-Plex manager 5.0 Software	Bio-Rad Labororatories Ltd, Hercules, CA, USA	
DC126291 Camera	Canon	
Eppendorf Biophotometer	Fisher Scientific, Loughborough, Leicestershire	
FACSDiva software version 7	Beckton Dickinson, Oxford, Oxfordshire	
FlowJo version 7	TreeStar, Inc., Ashland, OR	
Mr. Frosty™ freezing container	Thermo Scientific, Loughborough, Leicestershire	
Heraeus Biofuge primo Centrifuge	Thermo Scientific, Loughborough, Leicestershire	
Heraeus Pico17 mini Centrifuge	Thermo Scientific, Loughborough, Leicestershire	
Procell incubator	Jencons Scientific Ltd, Leighton Buzzard, Bedfordshire	
JB Aqua 26 plus water bath	Grant, Shepreth, Cambridgeshire	

LightCycler 480	Roche Diagnostics Ltd, West Sussex
LSRFortessa™ flow activated cell sorter	Beckton Dickinson, Oxford, Oxfordshire
Nanodrop spectrophotometer	Thermo Scientific, Loughborough, Leicestershire
Nucleocounter	ChemoMetec A/S Allerød, Denmark
Seven compact pH meter	Mettler-Toledo, Beaumont Leys, Leicestershire
Dry bath heating system	STARLAB (UK) Ltd, Milton Keynes, Buckinghamshire
TC10™ automated cell counter	BioRad, Hemel Hempstead, Hertfordshire
Trans-Blot® turbo™ transfer system	BioRad, Hemel Hempstead, Hertfordshire
Vortex genie 2	Scientific Industries, New York
Weighing scales	VWR, Lutterworth, Leicestershire

## **Preparation of solutions**

### **1M DL-Dithiothreitol (DTT)**

0.154 g DTT

1 ml distilled water

Storage: Aliquoted and stored at -20°C

### **1M Tris**

30.29 g Tris HCl

200 ml distilled water

pH as required

Storage: Room temperature

### **Dark Room Developer (Kodak®)**

210 ml Carestream® Kodak® autoradiography GBX developer

790 ml H<sub>2</sub>O

### **Dark Room Fixer (Kodak®)**

210 ml Carestream® Kodak® autoradiography GBX Fixer/replenisher 790 ml H<sub>2</sub>O

### **RNase (10mg/ml)**

100 mg RNase

10 ml PBS

Storage: Aliquoted and stored at -20°C

### **Propidium iodide (1mg/ml)**

10 mg Propidium iodide

10 ml PBS

Storage: Aliquoted and stored at -20°C

### **Running Buffer x10**

50 ml 20% Sodium dodecyl sulphate (SDS)

144 g Glycine

30 g Tris Base

Make up to 1 L with distilled water

Storage: Room temperature

**PBST x1**

10 phosphate buffered saline (PBS) tablets  
1 L distilled water  
1 ml Tween per 0.1% concentration desired  
Storage: Room temperature

**Reducing Sample Buffer x5**

1 g Sodium dodecyl sulphate (SDS) powder  
3 ml 1M Tris HCl pH 6.8  
5 ml Glycerol  
2 ml distilled water  
0.05 g bromophenol blue  
Storage: Aliquoted and stored at -20°C

**RIPA Buffer**

2 ml 1M Tris (pH7.5)  
5 ml 3M NaCl  
1 ml IGEPAL  
200 µL 0.5 EDTA  
91.8 ml dH<sub>2</sub>O  
Storage: Aliquoted and stored at 4°C  
1x Inhibitor tablet per 10ml (added when being used)

**10x Tris buffered saline**

87.66 g NaCl  
121 g Tris  
Make up to 1 l with distilled H<sub>2</sub>O  
pH to 7.5  
Storage: Room temperature

**Freeze media**

5 ml RPMI-1640 (10% FBS + 1% L Glutamine)  
500 µl DMSO

**Stripping Buffer**

10 ml SDS (20%)  
12.5 ml 1M TRIS HCL pH = 6.5  
700 µL Mecaptethanol  
76.8 ml H<sub>2</sub>O  
Storage: Room temperature



## 2.1 CELL CULTURE

The human pancreatic cell lines MIA PaCa-2, BxPC3, Panc-1 and Suit-2 were used. These cell lines were cultured at 37°C with 5% CO<sub>2</sub> in RPMI-1640 media that had 10% foetal bovine serum and 1% L-glutamine added. The cells were passaged every two to three days when at 80-90% confluency. A mouse cell line derived from a KPC mouse tumour was also used in this work. It was cultured at 37°C with 5% CO<sub>2</sub> in DMEM that had 10% foetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin and 1% amphotericin B. The cells were passaged every 2-3 days when at 80-90% confluency.

## 2.2 HARVESTING OF CELLS FOR OPTIMISATION OF WESTERN BLOT

A 90% confluent T75 flask of cells was used to generate cell lysates for optimisation of K-Ras detection by western blot. These cells were washed with PBS, which was then discarded. 10 ml of PBS was then added and the cells were scraped off the bottom of the flask using a cell scraper. The cell suspension was then transferred to a 15 ml falcon tube and spun at 1000 g for 10 minutes in a bench top centrifuge. The supernatant was removed ensuring that the cell pellet was not disturbed. The cell pellet was then stored at -80°C until cell lysates were made for western blotting.

## 2.3 HARVESTING OF CELLS FOLLOWING siRNA TREATMENT FOR WESTERN BLOT

Cells treated with siRNA were grown in 6 well plates see Table 1: . Each well had the media aspirated off and 1 ml of cold PBS added to the well. Using a cell scraper the cells were scraped off the bottom of the well. The cell suspension was then transferred to a 1.5 ml microcentrifuge tube and spun in a microcentrifuge for 10 minutes at 1000

g. After centrifugation the supernatant was removed, the contents of another well treated with the same conditions could then be added and the process repeated (if required). Once the cell pellet had been obtained it was stored at -80°C until cell lysates were prepared.

#### 2.4 **PREPARATION OF CELL LYSATES FOR WESTERN BLOT**

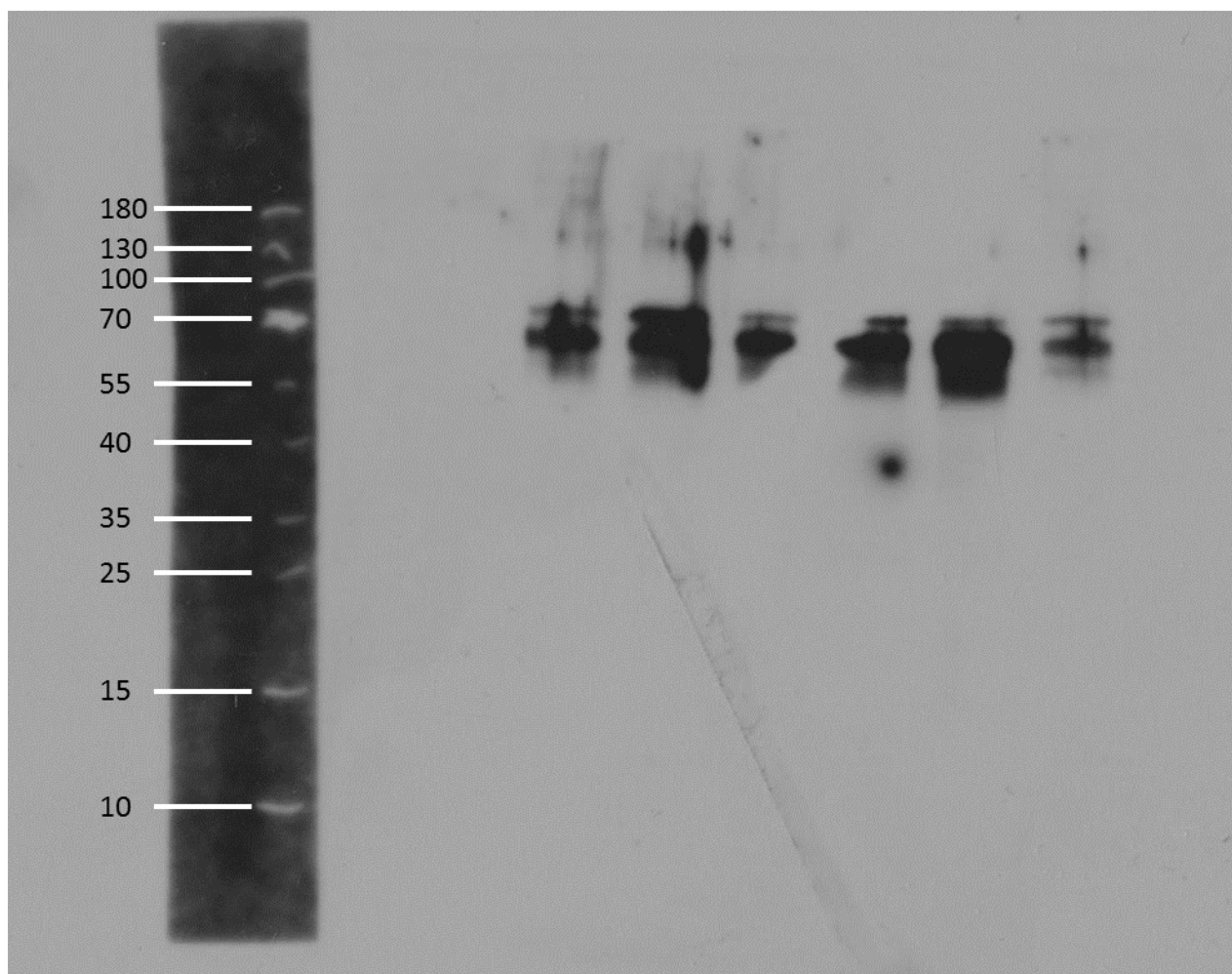
The cell pellets that had previously been frozen at -80°C were defrosted on ice before removing any excess liquid that remained without disturbing the pellet. The cell pellet was then resuspended in RIPA buffer and placed on ice. The suspension was then sonicated in a Branson Sonicade for 5 seconds at 30% output with continuous pulse. Following sonication the cells were kept on ice before being transferred to a microcentrifuge at 4°C and spun at 16434 g for 10 minutes. The supernatant was then transferred to a microcentrifuge tube and stored on ice.

#### 2.5 **QUANTIFICATION OF TOTAL PROTEIN USING THE BICINCHONINIC ACID ASSAY**

Protein was quantified using the BCA assay with the standards and working reagent being made up following the protocol in the BCA assay manual. Each sample to be measured was diluted 1:10 (5 µl in 45 µl of lysis buffer). 50 µl of each standard and each diluted sample was then added to 9950 µl of working reagent. These were then left at 37°C for approximately 25 minutes before being read on an Eppendorf bioPhotometer using the BCA micro program.

## 2.6 WESTERN BLOT

An identical quantity of protein was loaded into each well of a TGX AnyKD precast gel and a voltage of 300 volts was applied until the marker dye had separated suitably (approximately 15 minutes). Once run, the gel was removed from its plastic casing and placed on pre-prepared PVDF membrane. The sample was loaded into the Bio-Rad turbo blotter and transferred using the high molecular weight program pre-programmed by Bio-Rad (1.3 amp, 25 volts, and 10 minutes). Once transferred the membrane was blocked in 5% milk (non-fat blotting grade) in PBST (1x PBS, 0.1% Tween) for three hours rocking at room temperature. The primary antibody (diluted in 5% milk, 0.1%PBST) was added and left overnight rocking at 4°C. Following incubation with the primary antibody the membrane was washed three times for 10 minutes with PBST at 37°C. After washing, the secondary antibody (diluted in 5% milk, 0.1%PBST) was applied and left for 60 minutes rocking at room temperature. The membrane was washed six times for 10 minutes with warm (37°C) 0.1% PBST before being visualised using chemoluminescence and X-ray film. The position of the size markers on the western blot was recorded using tracker tape. Tracker tape is luminescent and marks the X-ray film allowing for the protein of interest to be compared to the size makers (Figure 2-1). If another antibody was to be tested the membranes were then stripped for 20 minutes at 50°C using harsh stripping buffer before being washed with distilled water. The same process as described above for the initial antibody was repeated for any further antibodies.



**Figure 2-1: Example western blot demonstrating how size markers were compared to the lanes of interest. Size markers positions on the western blot were recorded using tracker tape.**

## 2.7 siRNA TREATMENT OF CELL LINES

Cells were seeded into six well plates at the seeding densities shown in Table 1: and left over night in RPMI-1640 at 37°C with 5% CO<sub>2</sub>. After 24 hours the RPMI-1640 media was removed and replaced with a further 2.5 ml of RPMI-1640 media. 200 µl of Opti-MEM I containing 4 µl Lipofectamine 2000 and 200 µl of Opti-MEM I containing the siRNA Table 2: of interest were incubated at room temperature for 5 minutes before being mixed together and incubated for a further 30 minutes at room temperature. This solution was then added drop wise to each well and the plates were put at 37°C with 5% CO<sub>2</sub> until harvesting. Each knockdown experiment initially required 3 controls (although only the non-targeting control was used if they showed the same result in subsequent experiments), which are described in Table 3: .

Cell Line	Seeding density (cells/ml)
Suit-2	$1 \times 10^5$
Panc-1	$1 \times 10^5$
MIA PaCa-2	$2.5 \times 10^5$

Table 1: Table showing density that cells were seeded at in each well of a six well plate for siRNA experiments

<b>siRNA</b>	<b>Concentration</b>
K-Ras	10 nmol
N-Ras	100 nmol
Cyclin D	10 nmol
Ral A	10 nmol
Ral B	10 nmol

Table 2: Concentrations of siRNA used to treat cells seeded in a six well plate.

<b>Control</b>	<b>Type of control</b>
Lipofectamine only control	Control without any siRNA to show that the transfection procedure had no effect on the gene of interest
RISC free control	siRNA is chemically modified to prevent processing by the RISC complex.  Therefore, demonstrates any effects are not caused by the siRNA mechanism
Off target control	siRNA does not target any genes in the genome and therefore any effect on the gene of interest is due to off target effects

Table 3: Controls used for siRNA experiments

## 2.8 GROWTH CURVE EXPERIMENTS

Cells were set up in 6 well plates and knocked down using *KRAS* siRNA as previously described in section 2.7. For each time point to be examined 6 wells were set up containing the siRNA of interest and 4 wells were set up for each of the 3 controls (Lipofectamine only, Off target control and RISC free control). After 48hrs the cells were reseeded into T25 culture flasks and 10 ml of RPMI-1640 growth media was added. After 144 h the cells were split with a 1 in 4 dilution. This was done to prevent the cells overgrowing. The media was refreshed every 48 h when the cells had not been reseeded or split. Once set up 5 wells of the siRNA knockdown and 3 wells of each control were harvested for western blot analysis at 48, 96, 144, 192 and 216 hour time points following the same method as described in section 2.6. The final well of knockdown and the 3 controls were harvested and tested as described below.

### 2.8.1 HARVESTING AND TESTING CELL VIABILITY

The media from the well/flask was removed and spun down at 1000 g for 5 minutes. After centrifugation the supernatant was removed and the process was repeated until all the media had been spun down in the same microcentrifuge tube. Once all of the media had been removed 2 ml of PBS was added to the well/flask and left at 37°C for approximately 5 minutes. The PBS was then removed and spun down into the same microcentrifuge tube. 2 ml of trypsin was added to each well/flask and left at 37°C until all of the cells had detached. The cell/trypsin suspension was then spun down in the same microcentrifuge tube as the media and PBS previously, this allows for all the cells dead or alive to be collected from each well/ flask. The cell pellet from these spins was resuspended in 1 ml of media and 200 µl was used for the viable and nonviable cell

counts. To determine the viable and nonviable cell count the Nucleocounter was used. For the total cell count 200 µl of Nucleocounter solution A and B were added to the 200 µl of cells and then the solution was taken up into a Nucleocounter cassette and measured using the Nucleocounter. This was done in duplicate and the mean value was calculated. The nonviable cell count was determined using the 200 µl cell suspension, without any other solutions, this was also done in duplicate and an average taken. The Nucleocounter provides its cell counts in cells/ml and since only 1 ml was available per well no dilutions need to be accounted for. Therefore the percentage viability is determined using the formula:

$$\% \text{ viability} = \frac{\text{Average Total cell count} - \text{Average nonviable cell count}}{\text{Average Total cell count}} \times 100$$

## 2.9 FACS ANALYSIS OF SAMPLES

### 2.9.1 PROPIDIUM IODIDE TREATMENT OF CELLS FOR CELL CYCLE ANALYSIS

Samples were prepared in six well plates for FACS analysis, one well used per analysis. Knockdowns were done as described in section 2.7. The media was removed from the well and the cells were washed with 2x volume of PBS to remove any unattached cells. 300 µl of trypsin was added to each well and then left at 37°C until the cells detached. Once the cells have detached 1 ml of media was added and the cell suspension was transferred to a 15 ml conical tube. The cell suspension was centrifuged at 500 g (4°C) for 5 minutes and after centrifugation the supernatant was aspirated off. The cell pellet was washed with 1 ml of PBS and centrifuged at 500 g (4°C) for 5 minutes. The PBS was then aspirated off (apart from a few drops which were used to start the resuspension of the cell pellet) before adding 2 ml of ice cold ethanol drop wise; vortexing to ensure



that the cells were fully resuspended. The samples were then stored for a minimum of 16 hours at 4°C to allow the cells to fix (note they could have been stored for weeks with no detriment to the result).

On the day the FACS analysis was to be carried out the samples were centrifuged at 500 g (4°C) for 5 minutes and the supernatant aspirated and discarded. 500 µl of propidium iodide (50 µg/ml) and 50 µl of RNase (10mg/ml diluted 1:10 in PBS) were added to the sample before mixing by tapping gently and incubation in the dark at room temperature for 15 minutes. The sample was then transferred to a FACS tube and run on the FACS machine following the Good Clinical Laboratory Practice Facility SOPs GCLPEQU028/v1 and GCLPTSS092/v1. A minimum of 10000 cells were analysed using sequential gating. The 1<sup>st</sup> gate using forward scatter-area vs Forward scatter-height was to exclude doublets and clumps and the second gate was set on Forward scatter-area vs side scatter-area to exclude debris. The data was analysed using the software FlowJo version 7 and the analysis was performed on the cell cycle analysis platform using the Dean Jett Fox model. FlowJo offers two mathematical models to analyse cell cycle data. Both models fit Gaussian curves to G1 and G2. The Dean-Jett-Fox model fits S-phase to a second degree polynomial and the other model makes no assumptions on the shape of S-phase distribution. I tried both models and selected the Dean-Jett-Fox model for fitting the distribution of my cell cycle results as it gave a value for S-phase that fitted better with my impression of the raw histogram representations.

### 2.9.2 S-PHASE LABELLING OF CELLS USING EDU

Samples were prepared in six well plates for FACS analysis, one well used per analysis. The cells were treated with siRNA as described in section 2.7. Cells were treated with EdU (10mM) at either 24,26,28,30 or 32 h post siRNA treatment. The cells were then grown until 48 h after which the media was removed and 1 ml of PBS added to each well. The cells were scraped into the PBS before being transferred to a 15 ml falcon tube (15 ml falcon tubes rather than microcentrifuge tubes as this improved recovery of cells after subsequent centrifugation steps). The cells were spun at 1000 g for 10 minutes and the PBS was aspirated off. Each sample was washed with 1 ml 1% FBS in PBS before being spun again for 10 minutes (1000 g) and removing the supernatant. The pellets were then resuspended in 100 µl of Click-iT fixative (from the Click-iT® EdU Alexa Fluor® 647 flow cytometry assay kit) and incubated at room temperature for 15 minutes in the dark. The cells were then washed with 3 ml 1% FBS in PBS before being spun again for 10 minutes (1000 g) and then the supernatant was removed. The cell pellet was then resuspended in 100 µl of Click-iT saponin-based permeabilization and wash reagent and incubated for 15 minutes in the dark as before. While incubating during the previous step the Click-iT EdU reaction cocktail was prepared according to Table 4 . After the previous incubation has been completed 0.5 ml of the Click-iT EdU reaction cocktail was added to each sample, which was then incubated in the dark at room temperature for 30 minutes. The cells were washed once with 3 mls of the saponin-based permeabilization and wash reagent, spun for 10 minutes (1000 g) before resuspending the cells in 500 µl PI solution (propidium iodide (50 µg/ml) and 50 µl of RNase (10mg/ml diluted 1:10 in PBS) the cells were then incubated in the dark on ice for 15minutes. The sample was then transferred to a FACS tube and

run on the FACS following the Good Clinical Laboratory Practice Facility SOPs GCLPEQU028/v1 and GCLPTSS092/v1. A minimum of 10000 cells were analysed with appropriate gating. The analysis was performed using the FACS Diva version 7 software.

Reaction component	Volume for one reaction
PBS	438 µl
CuSO <sub>4</sub>	10 µl
Fluorescent dye azide	2.5 µl
Reaction buffer additive	50 µl
Total reaction volume	500 µl

Table 4: Reaction mixture for EdU staining.

## 2.10 PREPARATION OF CELLS FOR ANALYSIS BY THE SEAHORSE BIOANALYSER

Cells were seeded into the Seahorse specific 24 well plates Table 5: and left for 24 h at 37°C (5% CO<sub>2</sub>) before treatment with siRNA (if appropriate). After 24 h the media was removed and 0.5 ml of RPMI-1640 with 10% Foetal bovine serum and 1% L-glutamine was added to the wells. siRNA was prepared as previously described (see section 2.7) but only 15 µl of the siRNA mixture was added to each well. The cells were then left for 48 h at 37°C (5% CO<sub>2</sub>). The Seahorse cartridge to be used was hydrated the day before the run by adding 1 ml of Seahorse calibration solution to each well of a 24 well plate and leaving the seahorse cartridge to equilibrate overnight at 37°C with no CO<sub>2</sub>.

The media was removed from the wells and 450  $\mu$ l of Seahorse media was added (500 ml Bicarbonate free RPMI-1640 with 10mM glucose 2mM sodium pyruvate and 1% L-glutamine pH 7.4). The plate was then incubated for one hour at 37°C with no CO<sub>2</sub>. Each drug was diluted in Seahorse media before being loaded into the injection ports of the Seahorse cartridge Table 6: . The seahorse cartridge and plate were then added to the Seahorse Bioanalyser and run according to the programmed settings for an Aerobic stress test.

<b>Cell line</b>	<b>Number of cells seeded per well in a Seahorse 24 well plate</b>
Panc-1	12000
Suit-2	6000

Table 5: Number of cells to be seeded per well in a 24 well Seahorse Bioanalyser plate

<b>Drug</b>	<b>Concentration</b>	<b>Volume loaded into injection port</b>	<b>Injection port the drug was loaded in</b>
Oligomycin	1 $\mu$ g/ml	50 $\mu$ l	A
FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone)	0.5mM	55 $\mu$ l	B
Rotenone and Antimycin A (both drugs prepared together)	Both at 1 $\mu$ M	62 $\mu$ l	C

Table 6: Concentration and volume of drugs loaded into the injection ports of the Seahorse Bioanalyser

## 2.11 PREPARATION OF CELLS FOR LIPID ANALYSIS

Six well plates were seeded with Suit-2 cells. The cells were treated with *KRAS* siRNA at the previously optimised concentration and the controls were treated with the same concentration of control siRNA. The cells will then be left for 48 hours at 37°C 5% CO<sub>2</sub> before being harvested. To harvest the cells the media was aspirated off and the cells were washed with 3 ml of cold PBS before adding 1 ml of cold PBS to each well. The cells were then scraped into the PBS and transferred to a microcentrifuge tube, which was spun at 1000 g for 10 minutes. The supernatant was aspirated off and the process repeated until all the cells with the same treatment were pelleted in one microcentrifuge tube. The cells were then stored at -80°C before being sent to on dry ice to Professor Swinnen (Department of Oncology, University of Leuven) for lipid analysis.

## 2.12 PULLDOWN ASSAYS

### 2.12.1 ACTIVE RAS PULLDOWN

The samples were prepared in six well plates with 4 plates being required per condition to generate enough protein of subsequent western blot analysis. Knockdowns were done as in section 2.7 and left for 48hr at 37°C (5% CO<sub>2</sub>). Media was aspirated from the plates and 1 ml of cold TBS was added to each well. The cells were scraped into the TBS before pooling wells with the same knockdown conditions into a 50 ml Falcon tube which was then centrifuged at 1000 x g for 10 minutes. The supernatant was aspirated off and the cell pellet was resuspended in 1 ml of SDS free lysis buffer, which was supplied by the manufacturer. The sample was vortexed briefly before being left on ice for 15 minutes to lyse. The lysate was spun for 15 minutes at 4°C at 16434 g before the supernatant was transferred to fresh microcentrifuge tubes and kept on ice.

The protein concentration of the samples was determined using the BCA assay as describe in section 2.5.

For each sample 100 µl of 50% glutathione resin was added to a spin cup supplied by the manufacturer and then centrifuged at 6000 g for 30 seconds. The flow through was discarded and the spin cup was washed with 400 µl of lysis buffer before spinning again at 6000 g for 30 seconds and discarding the flow through. 80 µg of GST-Raf1-RBD was then added to the spin cup before 500 µl of lysis buffer containing 500 µg of protein was added. The sample was vortexed and incubated for 1 hour at 4°C with gentle rocking. After incubating for 1 hour the samples were centrifuged at 6000 g for 30 seconds and the flow through discarded. Each sample was washed 3 times with 400 µl lysis buffer centrifuging at 6000 x g for 30 seconds discarding the flow through between the washes. 50 µl of 2 x SDS Sample buffer was added to each spin cup and incubated for 2 minutes at room temperature before being centrifuged at 6000 g for 2 minutes into a fresh microcentrifuge tube. The spin cup was discarded and the sample stored at -20°C until it was to be analysed by western blot.

#### 2.12.2 UBIQUITIN PULLDOWN

The samples were prepared in six well plates with a minimum of four plates being required per condition. Knockdowns were done as in section 2.7 with 8 plates being prepared for each knockdown condition. The plates were incubated at 37°C (5% CO<sub>2</sub>). 24 hours post knockdown four plates from each condition were treated with bortezomib (10nM) or DMSO (equivalent amount to the volume used with the drug) in 500 µl of RPMI-1640 with 10% FBS. Cells were harvested 48 hours post siRNA treatment and lysed following the lysis protocol described in section 2.12.1. Once the protein lysate

had been prepared the concentration was determined using the BCA assay following the protocol in section 2.5.

For each sample 200 µg of protein was made up to 500 µl and was then added to a spin column. 20 µl of Polyubiquitin Affinity Resin from the ubiquitin pulldown kit was added to each sample and the samples were incubated at 4°C for two hours on a rotating wheel. After being left at 4°C the column was centrifuged for 15 seconds at 5000 g and the flow through was discarded. Three washes using 300 µl of wash buffer were performed centrifuging at 5000 g for 15 seconds and discarding the flow through after each spin. Each spin column was placed in a collection tube and 75 µl of sample buffer was then added. The samples were heated to 95°C for ten minutes before a final centrifuge step for one minute at 5000 g. The collection tubes were closed and stored at -20°C until they were to be analysed via western blot.

## 2.13 TRANSCRIPTIONAL ANALYSIS

### 2.13.1 PREPARATION AND HARVESTING OF SAMPLES

Cells were seeded (2 wells of a 6 well plate per condition) and treated with K-Ras, cyclin D or non-targeting siRNA as described in section 2.7. 48 hours post siRNA treatment the media was aspirated off and 1 ml of PBS was added to each well. The cells were scraped into the PBS and wells containing the same treatment were pooled. The samples were then centrifuged at 1000 g for 10 minutes before removing the supernatant and storing the pellet at -80°C until it was needed for RNA extraction.

### 2.13.2 PREPARATION OF RNA

The samples were prepared using an RNAeasy Mini Kit. 300 µl of buffer RLT was added to the cell pellet before spinning at 13300 g for three minutes. The supernatant was transferred to a microcentrifuge tube and 300 µl of 70% ethanol was added. The sample was then transferred to an RNAeasy spin column and centrifuged for 15 seconds at 8000 g. The flow through was discarded and the column was washed by adding 700 µl of buffer RW1 before spinning at 8000 g for 15 seconds. The process was repeated with 500 µl of buffer RPE and then again with another 500 µl of buffer RPE. After the second addition of RPE the sample was centrifuged for two minutes instead of 15 seconds at 8000 g. The column was then dried by discarding the flow through and re-spinning the column for one minute at 13300 g. The spin column was placed in a fresh collection tube and 50 µl of molecular biology grade water was added to the column. This was centrifuged at 8000 g for one minute and the elute was collected. Levels of RNA were quantified using a Nanodrop spectrophotometer and the samples were stored at -80°C until they were needed to make cDNA.

### 2.13.3 PREPARATION OF CDNA

The samples were prepared using the QuantiTech Reverse Transcription kit from the RNA prepared as in section 2.13.2. gDNA wipe out was assembled for each sample in a PCR tube following the recipe in Table 7: .



Reagent	Volume/ reaction
Wipe-out Buffer 7x	2 $\mu$ l
Total RNA	Up to 1 $\mu$ g
RNAse free water	Make up to 14 $\mu$ l
Total reaction volume	14 $\mu$ l

Table 7: Recipe for preparing the sample for DNA wipe-out treatment

The samples were then incubated at 42°C for two minutes before adding to the final reverse transcriptase mix following the recipe described in Table 8: . The samples were then incubated for 20 minutes at 42°C followed by three minutes at 95°C. The samples were then stored at -20°C until they were to be used for qPCR.

Reagent	Volume/reaction
Reverse transcription enzyme	1 $\mu$ l
Reverse transcription buffer 5x	4 $\mu$ l
Reverse transcription primer mix	1 $\mu$ l
Total volume of RNA mix	14 $\mu$ l
Total reaction volume	20 $\mu$ l

Table 8: Table showing the recipe for preparing the reverse transcriptase reaction

#### 2.13.4 qPCR

qPCR reactions are prepared as shown in Table 9: for each sample and includes a control that has the DNA template replaced with sterile H<sub>2</sub>O. Once prepared and loaded into a 96 well plate the samples were run on the Roche Light Cycler 480 for 50 cycles using the protocol in below Table 10:

GAPDH was used to normalise between samples with the delta Ct values being the comparator used between samples.

Reagent	Volume/ reaction
LightCycler 480 Sybr Green Master Mix	18 µl
H <sub>2</sub> O	7 µl
Primers	2 µl total (either 1 µl of forward and reverse primer or 2 µl if premixed)
cDNA from step 2.13.3	2 µl

Table 9: Recipe for preparing qPCR samples

	Step	Repetitions	Temperature	Time
Enzyme Activation	1	1	95°C	10 minutes
PCR cycle	2	50	95°C	15 seconds
			60°C	1 minute
Melt curve	3			

Table 10: PCR protocol used for Real-Time PCR.

## 2.14 MEASUREMENT OF CYTOKINES

Suit-2 cells were seeded in a 6 well plate and treated with *KRAS*, *NRAS*, *KRAS* with *NRAS* or Cyclin D siRNA as described in section 2.7 and then they were left for 48 hours at 37°C, 5% CO<sub>2</sub>. After 48 hours 1 ml of the media was aspirated and stored in a microcentrifuge tube at -80°C until it was required. The levels of 27 cytokines were measured using the Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay (referred to as: 27 plex), on the Bio Plex 200 System using the Bio-Plex Manager 5.0 software to perform the initial concentration measurements and data analysis. To perform the analysis 50 µl of serially diluted standards and a media blank were added the 27 Plex plate which contains magnetic antibody- coupled beads for each of the 27 analytes. To create the serially diluted standards a known amount of lyophilized standard was reconstituted with 500 µl of tissue culture medium. This was vortexed for 1-3 seconds and incubated on ice for 30 minutes. 8 microcentrifuge tubes were labelled 1-8 and 72 µl of culture media was added to microcentrifuge tube 1 with 50 µl of culture media being added to each of the remaining tubes. Following the 30

minute incubation on ice 128  $\mu$ l of stock standard was added to microcentrifuge tube 1. This was then vortexed briefly and 50  $\mu$ l was transferred to microcentrifuge tube 2, this process repeated with 50  $\mu$ l being added from the previous sample until microcentrifuge tube 8 was reached. Once the test samples were added the plate was incubated at room temperature under constant agitation (900 rpm) for one minute and then for 30 minutes at room temperature with agitation (300 rpm). The plates were washed on the Bio-Plex Pro Wash Station before adding 25  $\mu$ l of the secondary antibodies to each well and incubating at room temperature as before. After the incubation period the plate was again washed using the Bio-Plex Pro Wash station and then 50  $\mu$ l of Streptavidin-PE was added. The plate was then incubated at room temperature again but the 300 rpm step was changed to 10 minutes. Assay buffer (125  $\mu$ l) was added to each well and the samples were read on the Bioplex 200. The fluorescent intensities of each of the 27 cytokine, chemokines or growth factors were converted to pg/ml by comparing the obtained values to a standard curve.

## 2.15 STATISTICAL METHODS

All statistical analysis was performed using Statistical Package for the Social Sciences software (version 21.0 for Windows; SPSS, Inc, Chicago, Ill). Repeat measure data was tested using an independent sample T-test (significance quoted is 2-tailed).

## CHAPTER 3: RESULTS 1: ACTIVITY AND FUNCTIONS OF RAS

### ISOFORMS

*Hypothesis: no other Ras isoforms are active in pancreatic cancer cells*

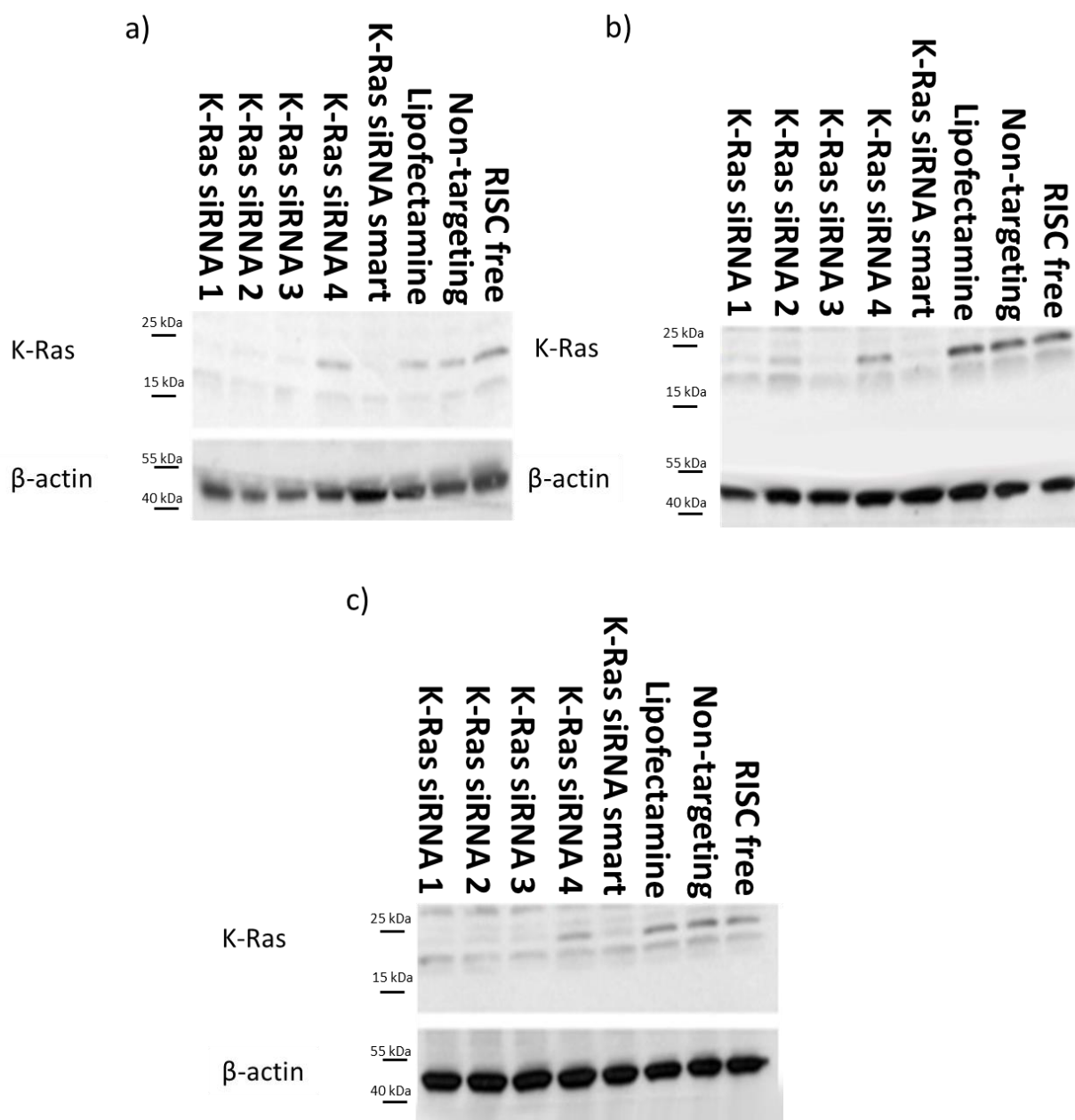
#### 3.1 K-RAS CAN BE DEPLETED IN PANCREATIC CANCER CELL LINES.

Mutant K-Ras is important in the formation of PDAC with evidence suggesting that some pancreatic cancer cell lines are addicted to it, and its loss results in cells undergoing cell death. (Singh et al., 2009) To identify if this was true for our cell lines, K-Ras was depleted using four different Dharmacon siRNAs (Figure 3-1). In all of the cell lines tested K-Ras was depleted using two of the four siRNAs (10nmol 48 h post transfection) with a third working in MIA PaCa2 and Panc-1 cells. The smart pool containing all four siRNAs together was used as a positive control and efficiently depleted K-Ras in all of the cell lines. From this data the siRNAs: K-Ras siRNA 1 and K-Ras siRNA 3 were the most efficient and worked in all cell lines tested. Therefore, all further K-Ras depletion experiments were done using K-Ras siRNA 1 unless otherwise stated.

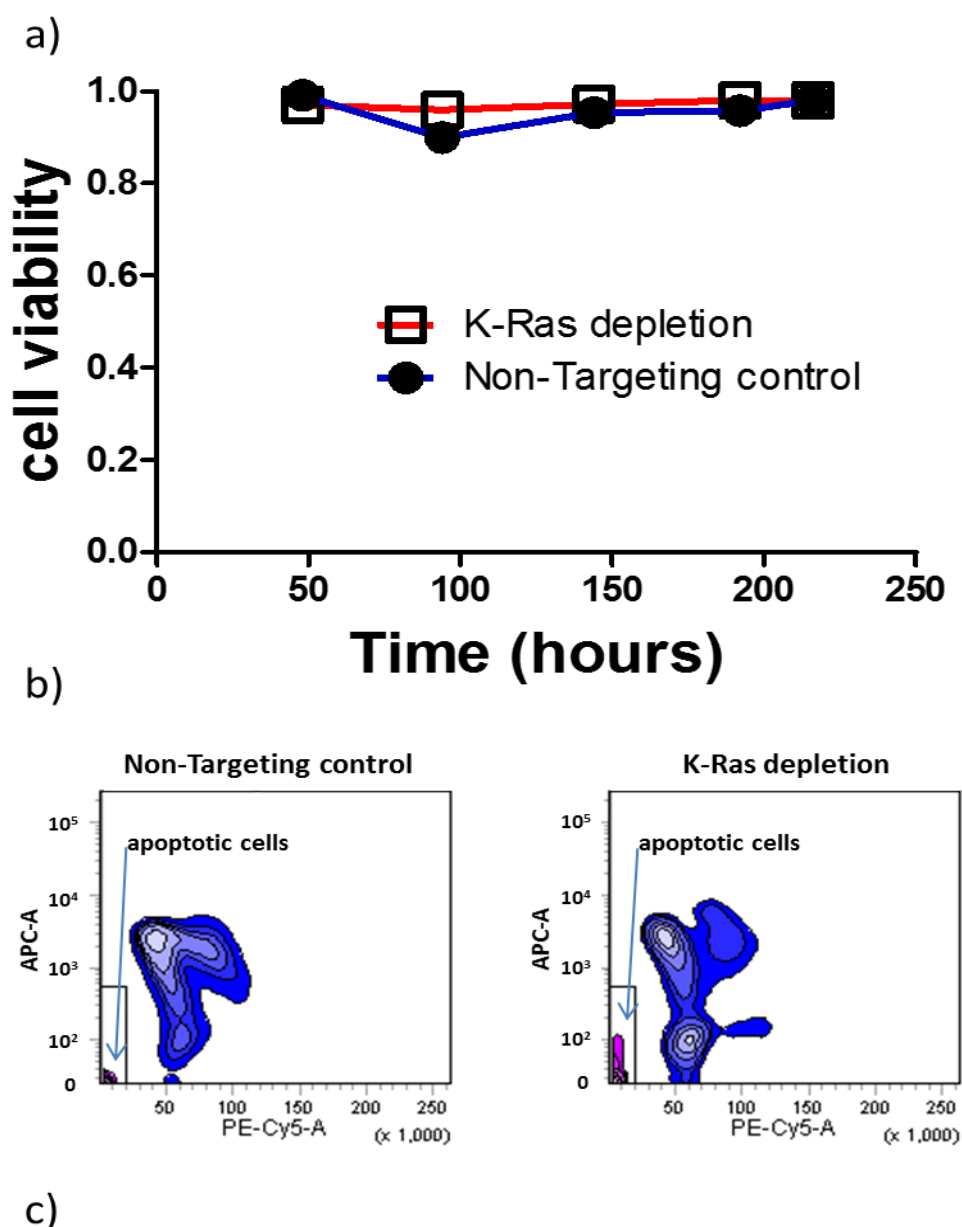
#### 3.2 K-RAS DEPLETION RESULTS IN VIABLE CELLS AND PROTEIN LEVELS WILL RETURN TO NORMAL SEVERAL DAYS AFTER siRNA TREATMENT

Suit-2 cells were treated with K-Ras siRNA and grown for several days following depletion. They were harvested and cell viability was tested (Figure 3-2). Depletion of K-Ras makes little or no difference to cell viability with both the controls and the K-Ras depleted cells having cell viabilities above 90%. Additionally, when cells were

examined using EdU and PI staining, the population of cells that was shown to be apoptotic only increases from 4 % in control to 7.9% in the K-Ras depleted cells (Figure 3-2). It can be seen in these cells as expected that the loss of K-Ras protein is only a transient effect. After 144 h the levels of K-Ras protein have increased and have returned to levels similar to the controls after approximately 192 h (Figure 3-3). While K-Ras is depleted cell growth is slowed and once K-Ras levels begin to recover the cells are able to grow more efficiently. The return of K-Ras to its pre-depletion levels results in the cells growing a similar manner to the control cells (Figure 3-4). Therefore we observe results that indicate that Suit-2 cells are able to survive the loss of K-Ras and continue growing once K-Ras is restored. This suggests that loss of K-Ras does not cause any permanent changes that are detrimental to cell growth.



**Figure 3-1: K-Ras depletion in pancreatic cancer cell lines. K-Ras was depleted in a) MiaPaCa2; b) Suit-2 and c) Panc-1 using 4 different K-Ras targeting siRNAs. K-Ras smart siRNA (all 4 siRNAs in a mixed pool) was used as a positive control along with 3 negative controls (no siRNA (Lipofectamine only), non-targeting siRNA and RISC free siRNA). K-Ras siRNAs 1, 2 and 3 were effective at depleting K-Ras in MIA PaCa2 and Panc-1 but, only 1 and 3 are effective in Suit-2 K-Ras siRNA 4 does not appear to be effective at depleting K-Ras in any of the cell lines.**



	% average apoptotic cells	Range of measurements %
Non-targeting control	4%	1.9%- 5.5%
K-Ras depleted cells	7.9%	4.7%-11.1%

Figure 3-2: Graphs showing viability of Suit-2 cells. a) K-Ras was depleted and the viability of the cells was tested up to 216 h post depletion using a nucleocounter (n=1). b) EdU representative FACS analysis showing a small increase in apoptotic cells following K-Ras depletion (n=3). c) Table showing average of FACS experiments in b) and the range of data measured.



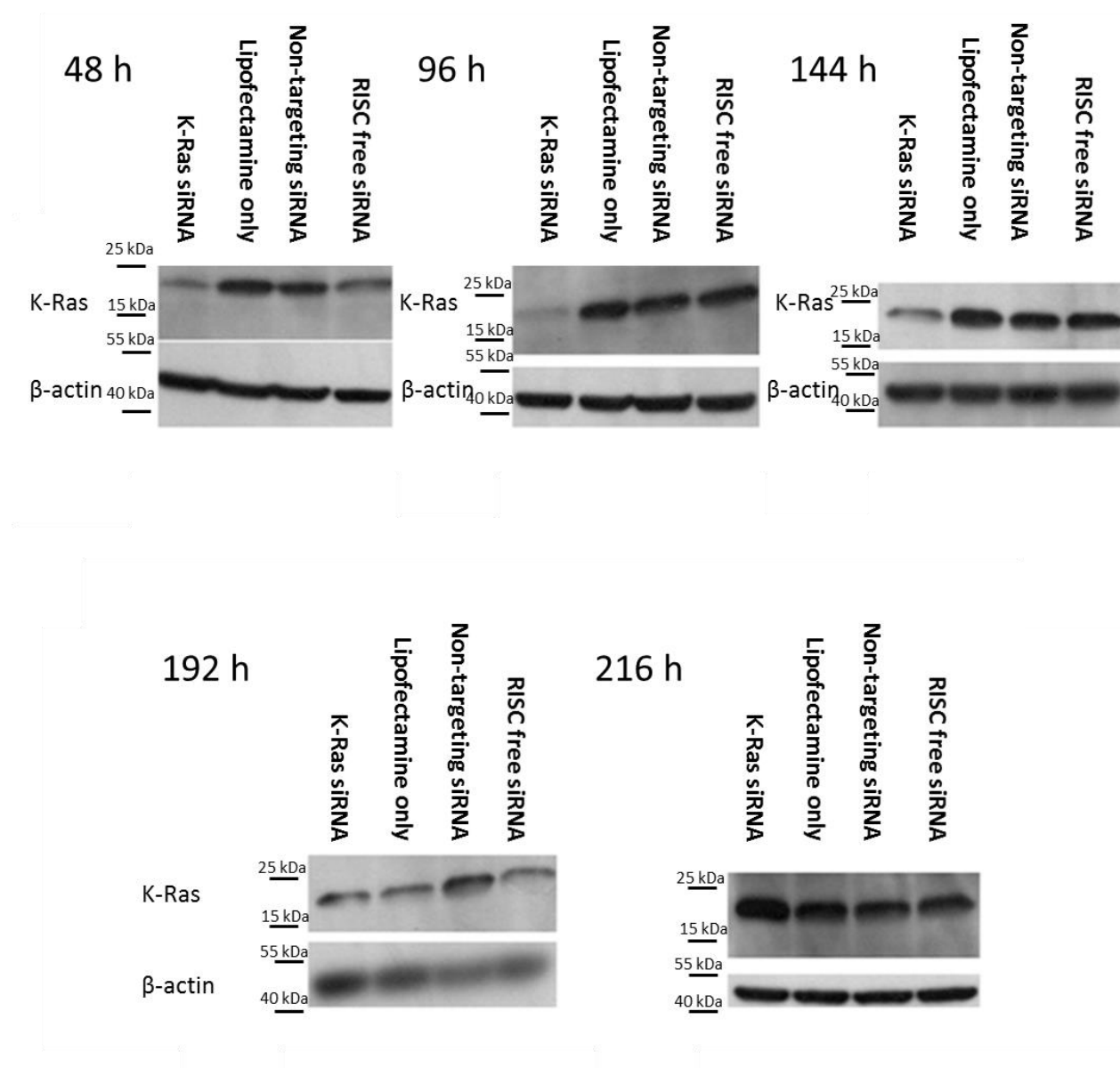


Figure 3-3: Western blot of Suit-2 cell time course demonstrating the level of K-Ras protein up to 216 h post siRNA treatment (n=1).

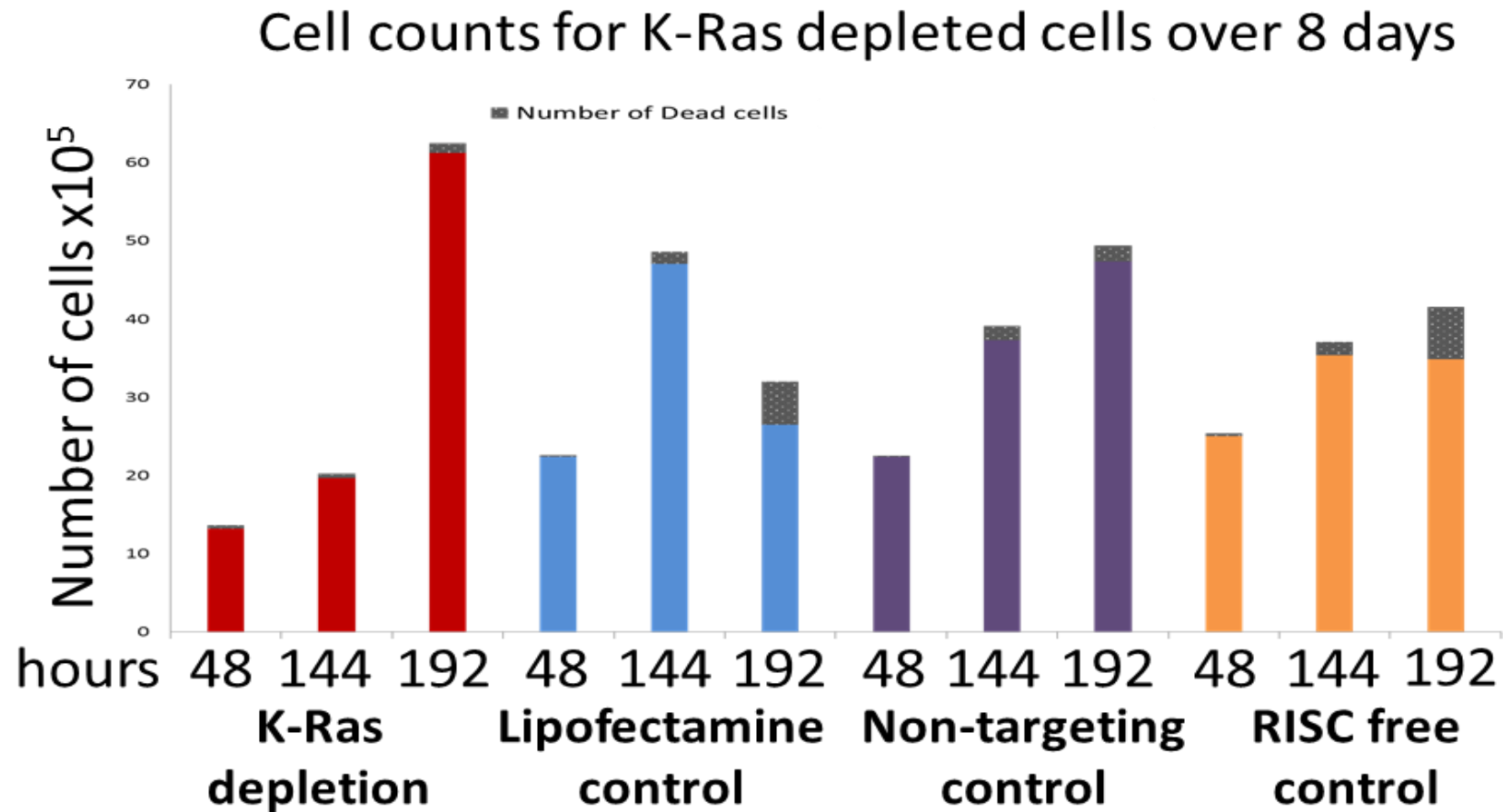
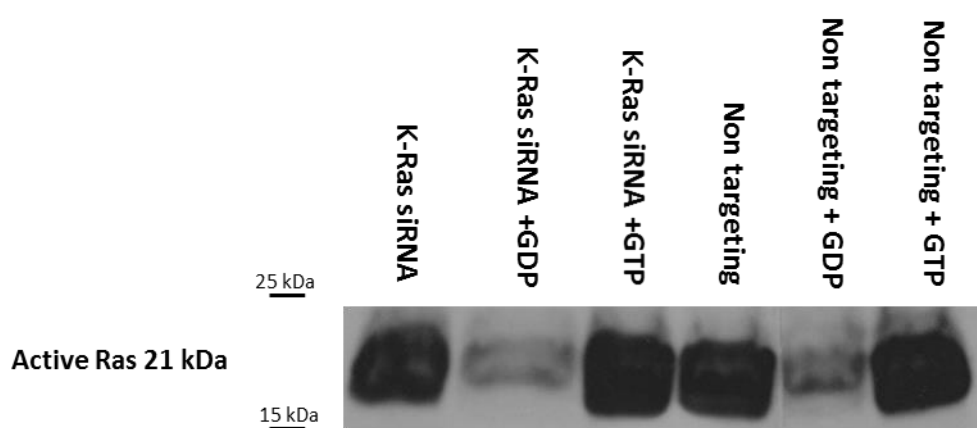


Figure 3-4: Cell numbers 48 hours 144 hours and 192 hours following K-Ras depletion (n=1). Cell numbers and viability were calculated using a nucleocounter (n=1).

### 3.3 ACTIVE RAS ISOFORMS IN PANCREATIC CANCER CELL LINES

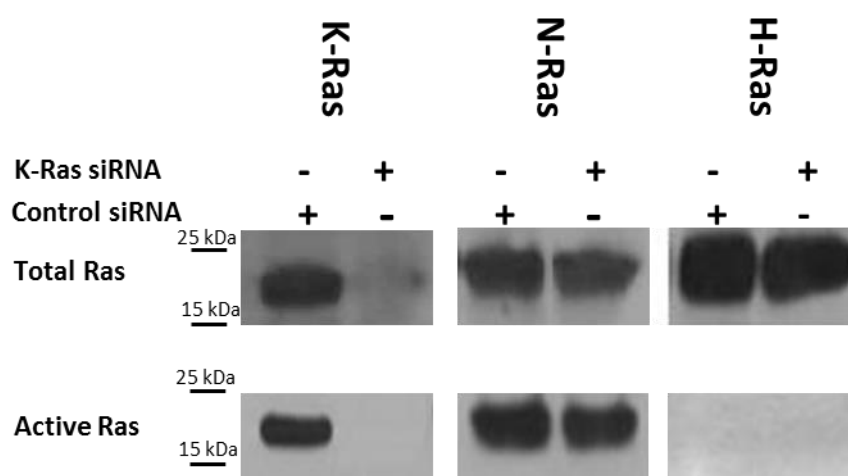
K-Ras is mutant and is expected to be active in our cell lines. This activity was tested by using an active Ras pulldown kit, which used Raf as a substrate to detect Ras activity. Once Ras had been pulled down using Raf, we probed using a pan Ras antibody supplied in the kit (Figure 3-5). GTP was added to activate Ras (GTP positive control) and GDP to give inactive Ras (GDP negative control). The pan Ras antibody detected Ras protein in the non-targeting control, with no Ras being detected in the GDP negative control and Ras being detected in the GTP positive control. When we looked at the K-Ras siRNA treated cells however, the cells that are depleted for K-Ras showed Ras to still be present and this band was lost in the same lysate treated with GDP, suggesting that the band is active Ras protein. This data not only confirms previous observations that other Ras isoforms are present in Suit-2 cells, (Omerovic et al., 2008) but also shows evidence suggesting that another Ras isoform is active via Raf.



**Figure 3-5: Active Ras in Suit-2 cell lines. Active Ras was isolated using Raf from Suit-2 cells treated with either K-Ras siRNA or control. For each siRNA one sample was pre-treated with GDP negative control, GTP positive control or no pre-treatment was applied. A pan Ras antibody from the kit was used to detect the presence of Ras protein (n=1).**

To further examine this I probed Suit-2 cells, which had K-Ras depleted, using antibodies against all three Ras isoforms (K, N and H). Looking at total Ras, which includes active and inactive forms, it can be seen that all three isoforms are present, (Figure 3-6) thus confirming what had previously been reported. Examination of only active Ras from our lysates showed that both K-Ras and N-Ras were present but H-Ras is not (Figure 3-6). This demonstrates that in the Suit-2 cell line all three Ras isoforms are present, but only K and N-Ras are active via Raf. Further examination of BxPC3, Panc-1 and a KPC mouse derived cell lines showed that they also contain both K and N-Ras. The human derived cell lines also have active K and N-Ras, but the KPC derived cell line does not appear to have much, if any, active N-Ras activity (Figure 3-7).

I have demonstrated that human pancreatic cancer cell lines have some N-Ras activity but in contrast a cell line, derived from the KPC mouse model, appears to be lacking this N-Ras Raf binding activity (Figure 3-7). Additionally it does not appear that N-Ras levels are dependent on K-Ras (at least in Suit-2 cells) because depletion of K-Ras appears to have no effect on the level of N-Ras protein present (Figure 3-6).



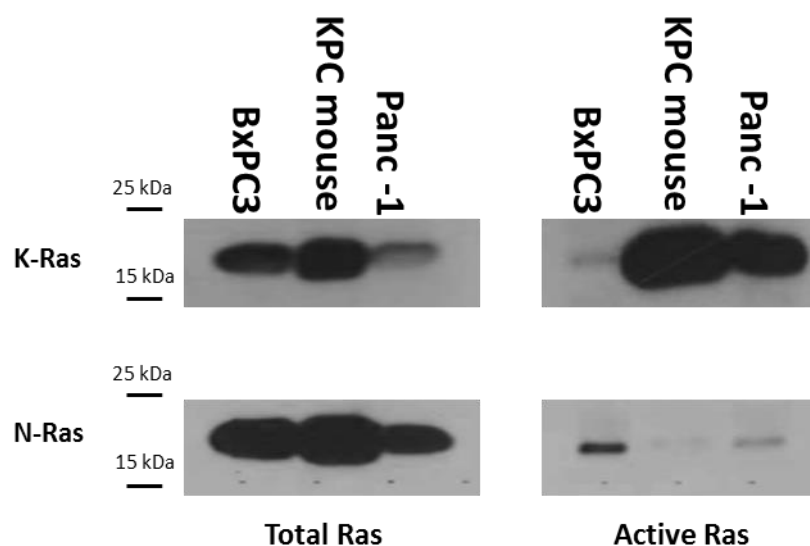
**Figure 3-6: Total and active Ras isoforms in Suit-2 cells. Western blot analysis showing the presence of each Ras species, either total Ras or active Ras isolated by Raf pulldown. The western blot is representative of the observed results (n=3).**

### 3.4 N-RAS FUNCTIONS IN HUMAN PANCREATIC CANCER CELL LINES

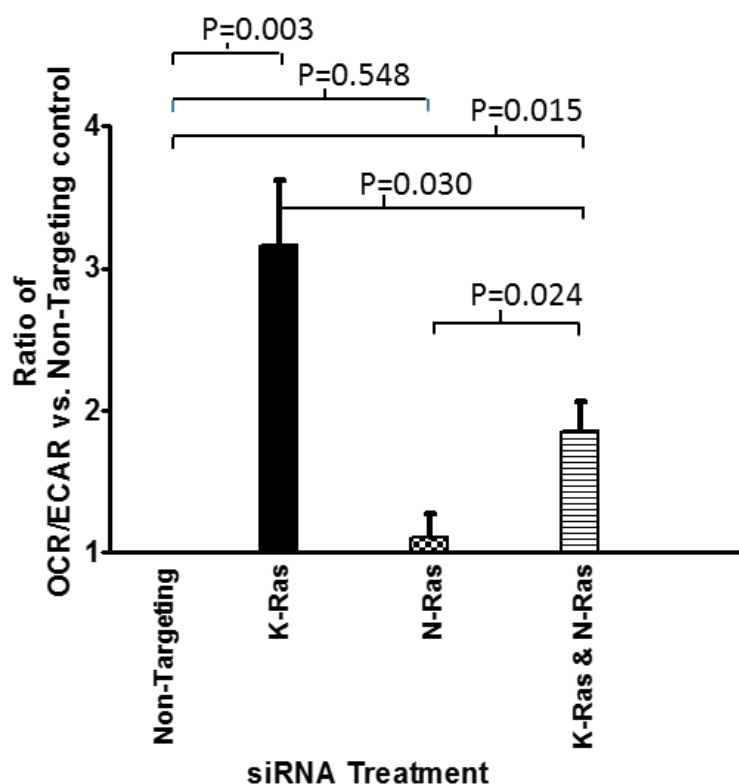
#### 3.4.1 RAS ISOFORMS MODIFYING GROWTH PATTERNS

N-Ras was shown to be active in all three of the human cell lines initially tested (Figure 3-6 and Figure 3-7), although a derivative of one of the cell lines was later identified without this active protein (see below). Using siRNA, N-Ras was depleted alone or in conjunction with K-Ras. To determine possible functions for N-Ras, both Ras isoforms were depleted and their growth patterns examined using the Seahorse Bioanalyzer (Figure 3-8). Mutant K-Ras has previously been demonstrated to be involved in reduction of oxidative phosphorylation in cancer cells. (Ying et al., 2012) The loss of K-Ras results in a shift towards oxidative growth, as shown in Figure 3-8 however, N-Ras depletion, does not appear to result in any significant change in the levels of oxidative vs non oxidative growth. When both are depleted together an intermediate shift towards oxidative growth is observed and this shift is significantly different from both K-Ras

depletion alone and the non-targeting control (Figure 3-8). This suggests that N-Ras has a function that is the opposite of K-Ras, but its loss alone is not significant to alter the levels of oxidative phosphorylation in Suit-2 cells.



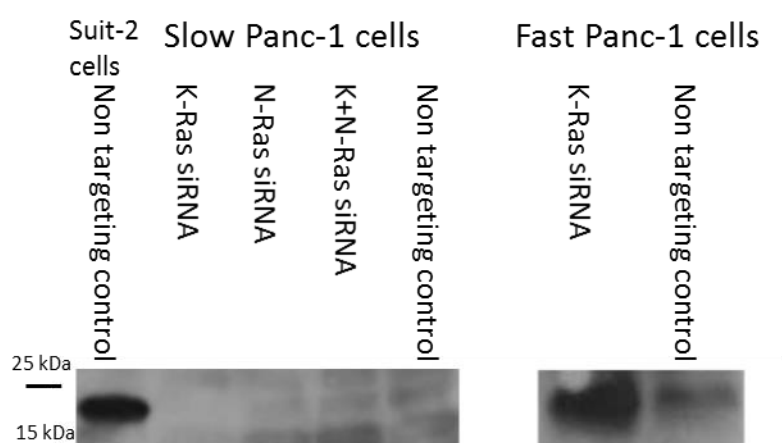
**Figure 3-7: Total and active Ras isoforms in pancreatic cancer cell lines. Representative western blot analysis (n=3) to demonstrate the presence or absence of total or active Ras isolated by Raf pulldown. The name of each cell line tested is given above the blot (n=3).**



**Figure 3-8: Effect of K-Ras and N-Ras on oxidative phosphorylation in Suit-2 cells.** The analysis of the Seahorse Bioanalyzer data shows how the oxygen consumption rate (OCR) vs extracellular acidification rate (ECAR) changes with the depletion of K-Ras  $n=7$ , N-Ras  $n=5$  or both  $n=5$ . K-Ras causes the greatest shift towards OCR, N-Ras gives little change and K-Ras with N-Ras gives an intermediate result when compared to the non-targeting control.

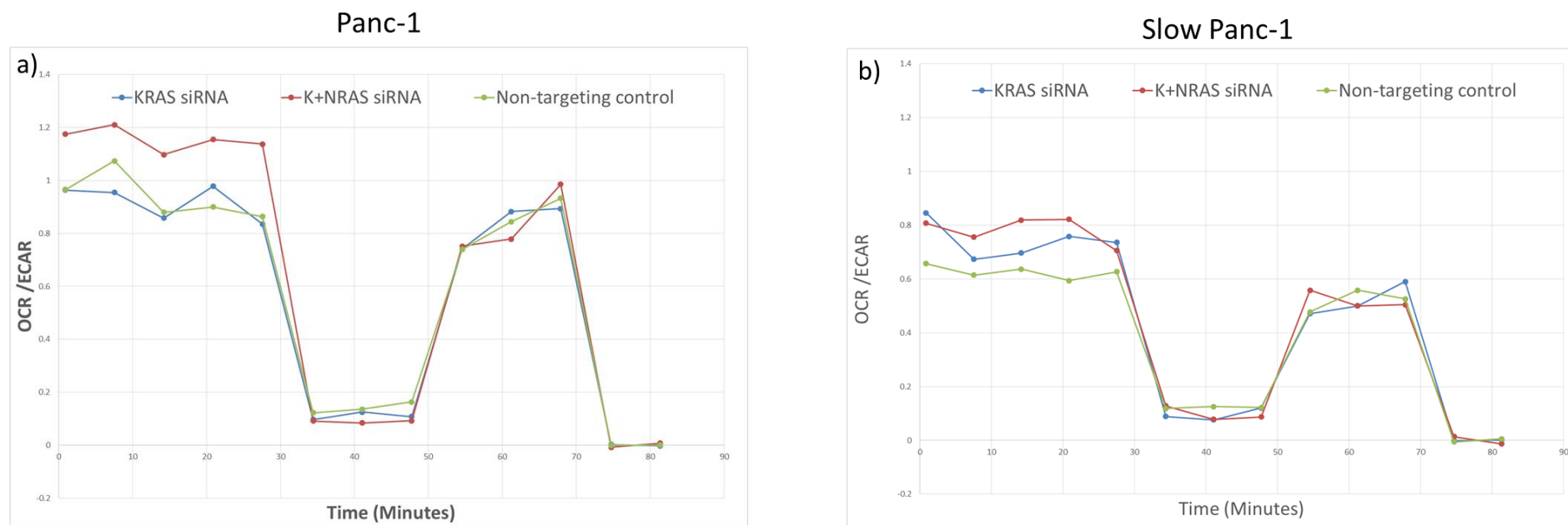
During this project I identified a slower growing Panc-1 cell line with a doubling time of approximately 48 hours compared to the normal doubling time of 24hours (defined as Panc-1 slow). Both the regular and slow Panc-1 cell lines were obtained from the ATCC at different times and both genotyped as Panc-1 cells. Genotyping was done independently using the PowerPlex16HS kit and compared to the markers provided by the ATCC. Western bolt analysis of Panc-1 slow cells suggests that they do not appear to have any active N-Ras present but the faster growing Panc-1 cell line does (Figure 3-9, Figure 3-7). The growth of these cells was also analysed using the Seahorse

Bioanalyzer. Both of the cell lines showed similar patterns of oxidative vs non oxidative growth when untreated (Figure 3-10). But when K-Ras was depleted alone in the regular Panc-1 cell line it did not appear to result in a change in growth: both K and N-Ras had to be depleted before a change to oxidative growth was observed (Figure 3-10). By contrast the Panc-1 slow cells that did not have active N-Ras demonstrated a similar shift towards oxidative growth when K-Ras alone or both K and N Ras were depleted (Figure 3-10). This result suggests that N-Ras may have developed a function in these cells that is overlapping with that of K-Ras. In the cells where N-Ras is not normally active however, K-Ras appears to be capable of maintaining the balance of oxidative vs non oxidative growth on its own.



**Figure 3-9: Active N-Ras presence in two different Panc-1 cell lines. Two different Panc-1 cell lines were tested for active N-Ras (n=1).**





**Figure 3-10: Aerobic stress test of two Panc-1 cell lines with different dividing times. Seahorse Bioanalyzer aerobic stress tests to determine the ratio of OCR vs ECAR. a) Panc-1 cells (n=1). b) Slow Panc-1 cells (n=1).**

### 3.4.2 RAS ISOFORMS INDEPENDENTLY ALTER CYTOKINE EXPRESSION PATTERNS

The effect of N-Ras depletion on oxidative phosphorylation is evidence that N-Ras has a function in our pancreatic cell lines. This evidence allows us to reject our hypothesis that no other Ras isoforms are active but, the impact in this respect does appear small. To obtain further evidence related to our original hypothesis and demonstrate functional roles for N-Ras in pancreatic cancer cell lines, an impact on the secretions from the cell was investigated. These secretions represent a form of finger print for the cell and any alteration to this fingerprint would represent a reporter for functional N-Ras activity. To examine this, the commercially available Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay kit was used. I depleted K-Ras and N-Ras alone or in conjunction with each other and the levels of cytokines in the supernatant were examined. Of the 27 cytokines that were examined using the assay only five had detectable levels in the supernatant Table 11: these five cytokines were then used to analyse the differences between K-Ras and N-Ras. The loss of N-Ras results in a relatively higher expression of IL-8 and a lower relative expression of IL-12 (Figure 3-11). This was not replicated when K-Ras is depleted: IL-12 exhibited an increase (Figure 3-11) and the depletion of both N-Ras and K-Ras resulted in a pattern closer to K-Ras than N-Ras. This therefore potentially shows a function of N-Ras but its significance is unclear.

<b>Cytokine</b>	<b>Detectable in supernatant (Yes or No)</b>
PDGF-bb	No
IL-1b	No
IL-1ra	No
IL-2	No
IL-4	No
IL-5	No
IL-6	No
IL-7	No
<b>IL-8</b>	<b>Yes</b>
IL-9	No
<b>IL-10</b>	<b>Yes</b>
<b>IL-12</b>	<b>Yes</b>
IL-13	No
IL-15	No
IL-17	No
Eotaxin	No
FGF basic	No
G-CSF	No
<b>GM-CSF</b>	<b>Yes</b>
IFN-g	No
IP-10	No
MCP-1(MCAF)	No
MIP-1a	No
MIP-1b	No
RANTES	No
TNF- $\alpha$	No
<b>VEGF</b>	<b>Yes</b>

Table 11; Table showing cytokines in the Bio-Plex Pro 27 Plex Human Cytokine, Chemokine and Growth Factor Assay kit. All of the cytokines in the Bio-Plex Pro 27 Plex Human Cytokine assay are listed. The ones highlighted in bold were the ones that had detectable levels and were taken for further analysis.

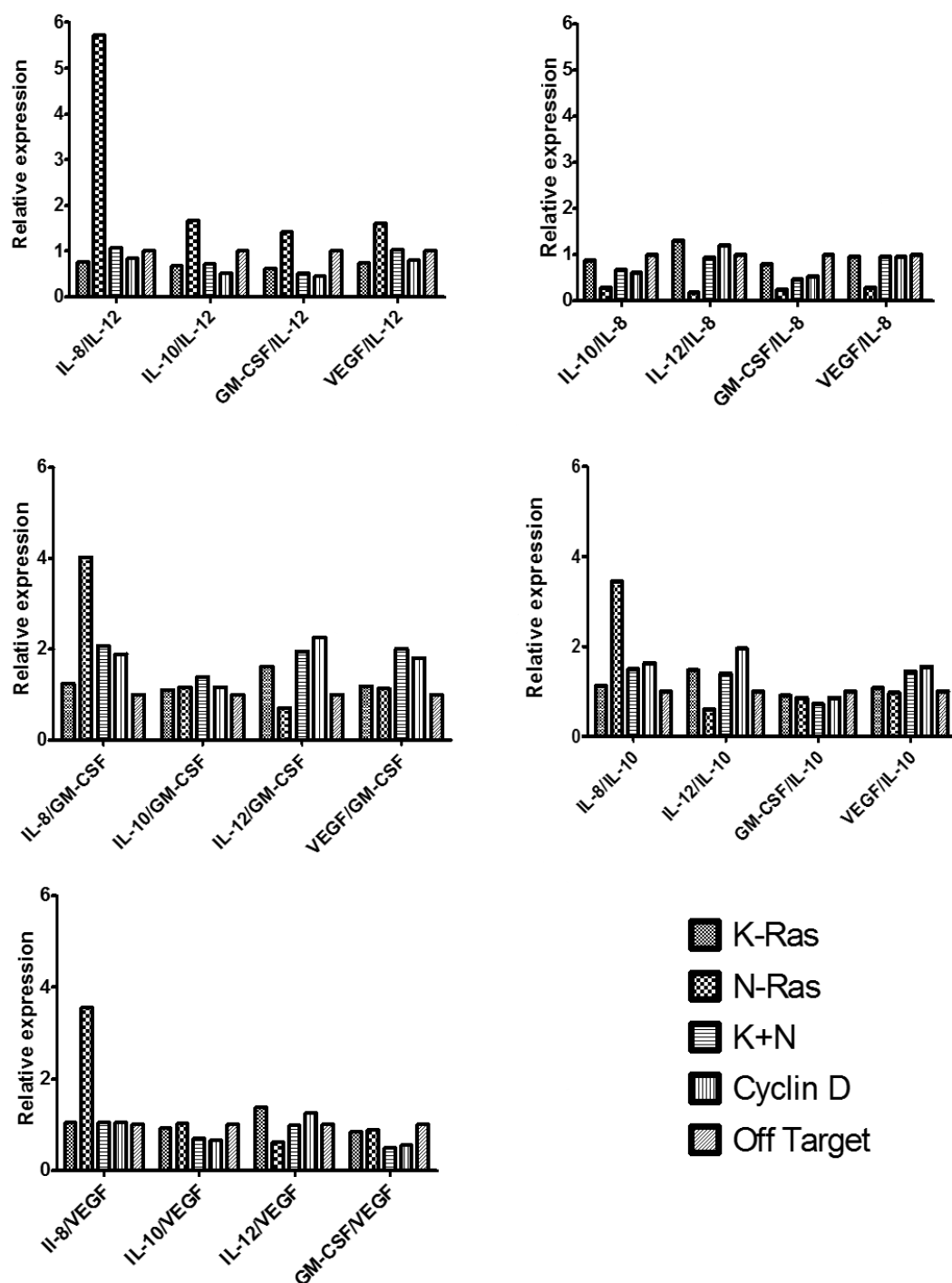


Figure 3-11: Effect of K-Ras and N-Ras on the cytokine expression profile of Suit-2 cells. The graphs show the changes in relative cytokine levels when Suit-2 cells were treated with K-Ras, N-Ras or Non-targeting siRNA (n=1).

### 3.4.3 MEMBRANE LIPID COMPOSITIONS ARE MODIFIED UNIQUELY BY K-RAS AND N-RAS

Ras is known to influence membrane composition as described previously, but the exact pattern of change will depend on many factors. Suit-2 cells were used to examine how the depletion of K-Ras and N-Ras effected the lipid composition of membranes. This analysis was carried out on a single sample for each depletion and compared to cells treated with non-targeting control siRNA. The loss of K-Ras results in a general increase in the more unsaturated phosphatidylcholines (>4 double bonds) and a decrease in the less unsaturated ones (<4 double bonds) (Figure 3-12). N-Ras depletion however, results in mostly small decreases in abundance with some of the medium length chains showing a slight increase (Figure 3-12). The combination treatment results in a pattern similar to the K-Ras depletion alone but with smaller changes (Figure 3-12) suggesting that N-Ras is perhaps responsible for some small modulation of change in the types of phosphatidylcholine present but K-Ras is the effector.

When phosphatidylethanolamine was examined the general trend when K-Ras was depleted was an increase in the levels of the longer chain lipids (most containing more than 36 carbons) with the shorter chain moieties decreasing in abundance (Figure 3-13). N-Ras depletion shows a similar pattern but once again the level of changes was generally smaller (Figure 3-13). Depleting with both K and N-Ras again results in a lipid profile that is similar to the K-Ras depletion (Figure 3-13) suggesting again that K-Ras is the major effector of the phosphatidylethanolamine expression profile.

K-Ras depletion resulted in a decrease in phosphatidylinositol levels, both in lipids containing 2 double bonds or less or more than 7 double bonds (plus the longest chain

with 6 double bonds). The lipids with unsaturation levels between these high and low points mostly showed increased levels when K-Ras was depleted (Figure 3-14). N-Ras depletion did not show any obvious pattern when it was depleted and most of the changes in relative abundance were small (Figure 3-14). When both were depleted the pattern was similar to that seen with K-Ras. Although, it was noted that the phospholipids with 7 double bonds appear to increase in number instead of the large decrease seen in the 38 and 40 carbon length chains of the K-Ras depletion (Figure 3-14).

Phosphatidylserine demonstrated no obvious pattern with some lipids being increased and others being decreased following K-Ras depletion (Figure 3-15). When N-Ras was depleted there seems to be more of the phospholipids decreasing in abundance than increasing (Figure 3-15). When both were depleted the pattern observed was much closer to K-Ras than N-Ras (Figure 3-15).

Thus, although N-Ras is functionally active in these cells (as shown in Figure 3-6 and Figure 3-7) this data indicates that the lipid profile is determined by K-Ras.

Treating Suit-2 cells with inhibitors of PI3K (GCD-0941) and MAPK (PD0325901) changed the relevant abundance of phosphatidylcholine compared to the control. Both inhibitors result in an increase in the abundance of the more unsaturated (>4 double bonds) although PD0325901 treated cells have a decrease in phosphatidylcholine in fatty acid chains with 10 double bonds or greater (Figure 3-16). Depletion of K-Ras (Figure 3-12) shows similar patterns of changes in abundance to the cells that were treated with either one of the inhibitors. However, the pattern of expression that was most similar was when Suit-2 cells were treated with both inhibitors at the same time.

This is perhaps surprising as Raf has previously been shown to have little effect on the Kennedy pathway (Ramirez de Molina et al., 2002).

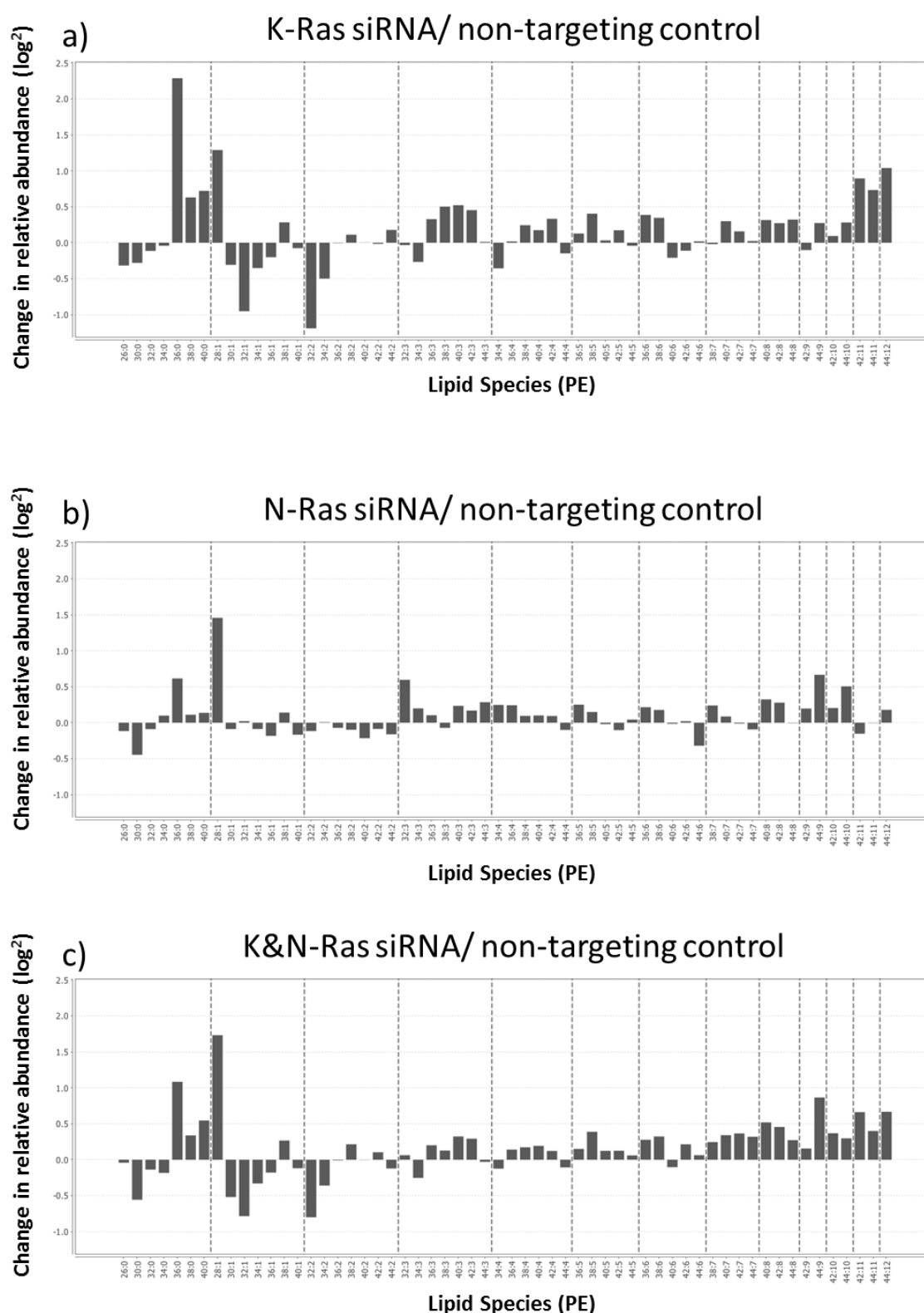
Phosphatidylethanolamine patterns in PD0325901 treated cells (Figure 3-17) were stable. However, treatment with GCD-0941 caused an increase in many of the phospholipids (Figure 3-17). Combination treatment much more closely resembled PI3K inhibition than MAPK (Figure 3-17). K-Ras depletion (Figure 3-13) resembled PI3K inhibition although the amount of phosphatidylethanolamines with 2 or 4 double bonds appears to change differently.

PD0325901 treatment results in phosphatidylinositol showing small changes from the control (Figure 3-18). GCD-0941 treatment shows greater changes (Figure 3-18) and these changes were similar to those observed when both PI3K and MAPK (Figure 3-18) were inhibited together or K-Ras alone was depleted (Figure 3-14).

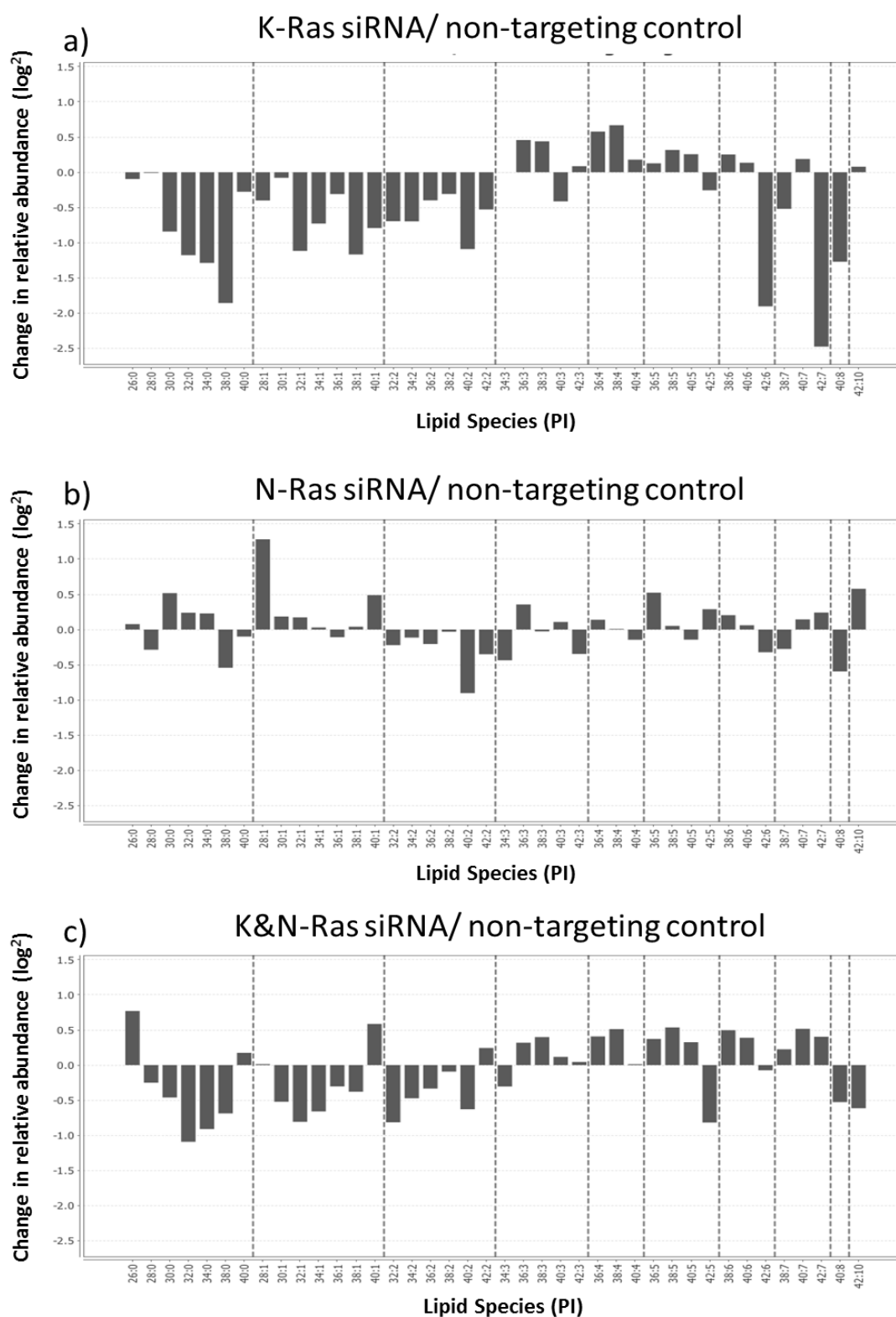
Finally, phosphatidylserine was examined and once again the individual depletion of PI3K closely mirrored both the double drug treatment (Figure 3-19) and the K-Ras depletion (Figure 3-15). Therefore, this evidence suggests that the PI3K pathway is most likely to be involved in the determination of lipid membrane profiles and is perhaps the pathway most closely associated with K-Ras regulation of membrane lipids. Other pathways or explanations cannot be excluded however, including changes to cell cycle profile.







**Figure 3-13: Membrane lipid analysis phosphatidylethanolamine. Relative abundance of phosphatidylethanolamine and phosphatidylethanolamine species in Suit-2 cells when K-Ras, N-Ras or both are depleted compared to non-targeting control siRNA. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group (n=1).**



**Figure 3-14: Membrane lipid analysis phosphatidylinositol. Relative abundance of phosphatidylinositol and phosphatidylinositol species in Suit-2 cells when K-Ras, N-Ras or both are depleted compared to non-targeting control siRNA. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group (n=1).**

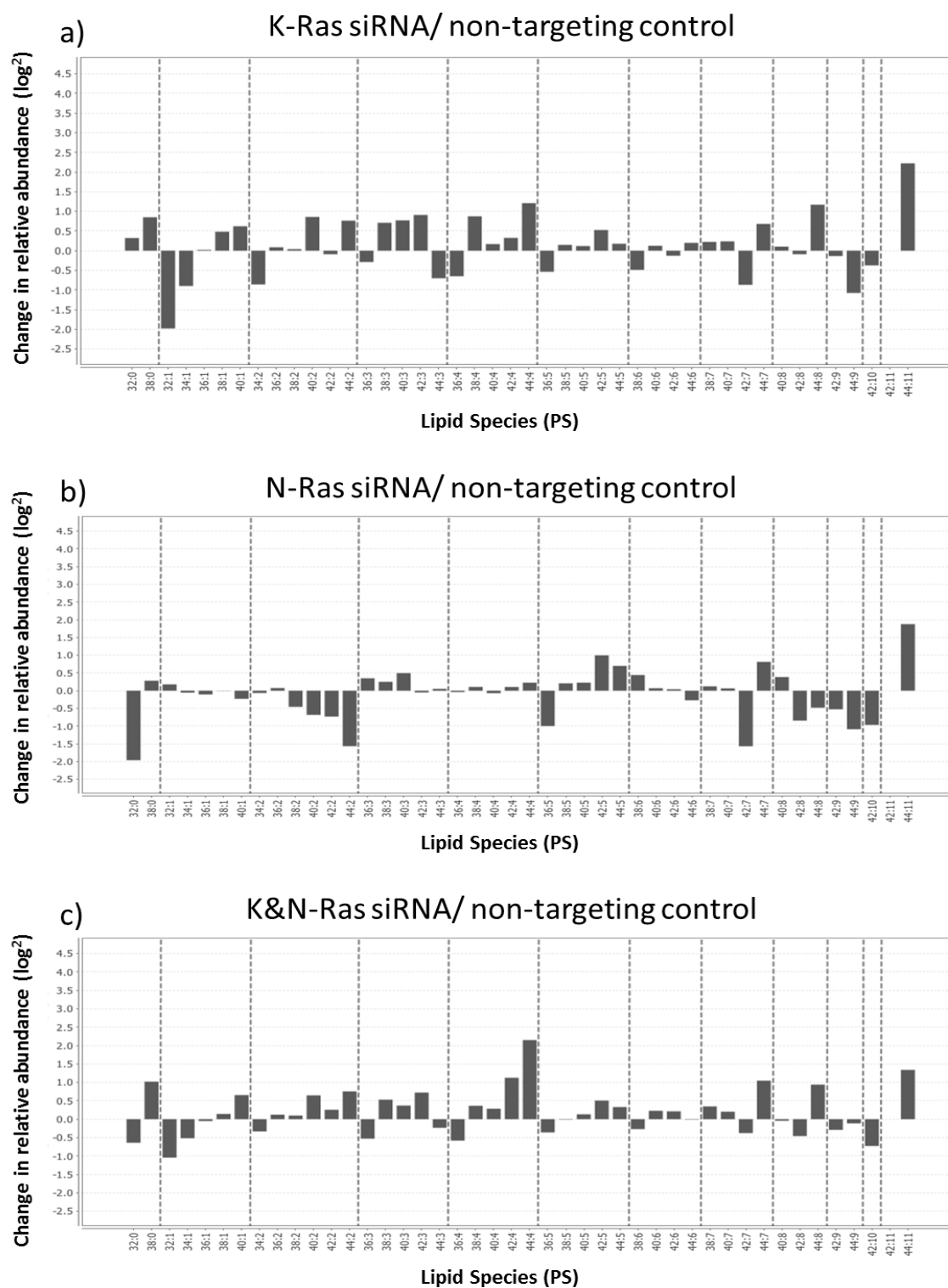


Figure 3-15: Membrane lipid analysis phosphatidylserine. Relative abundance of phosphatidylserine and phosphatidylserine species in Suit-2 cells when K-Ras, N-Ras or both are depleted compared to non-targeting control siRNA. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group ( $n=1$ ).

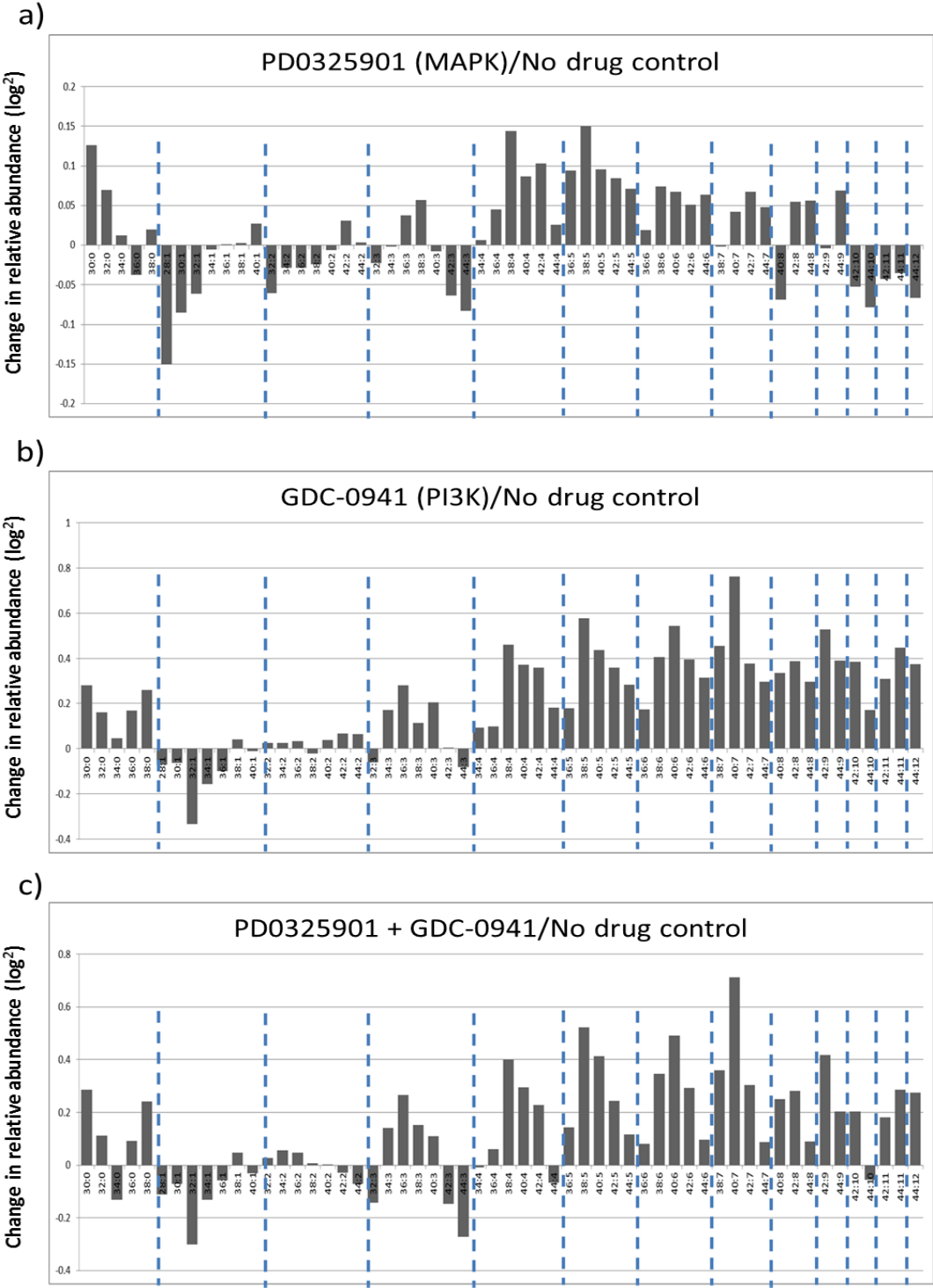


Figure 3-16: Membrane lipid analysis of phosphatidylcholine levels when cells are treated with MAPK and PI3K inhibitors. Relative abundance of phosphatidylcholine and phosphatidylcholine species in Suit-2 cells when the cells are treated with MAPK, PI3K inhibitors either alone or together. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group (n=1).

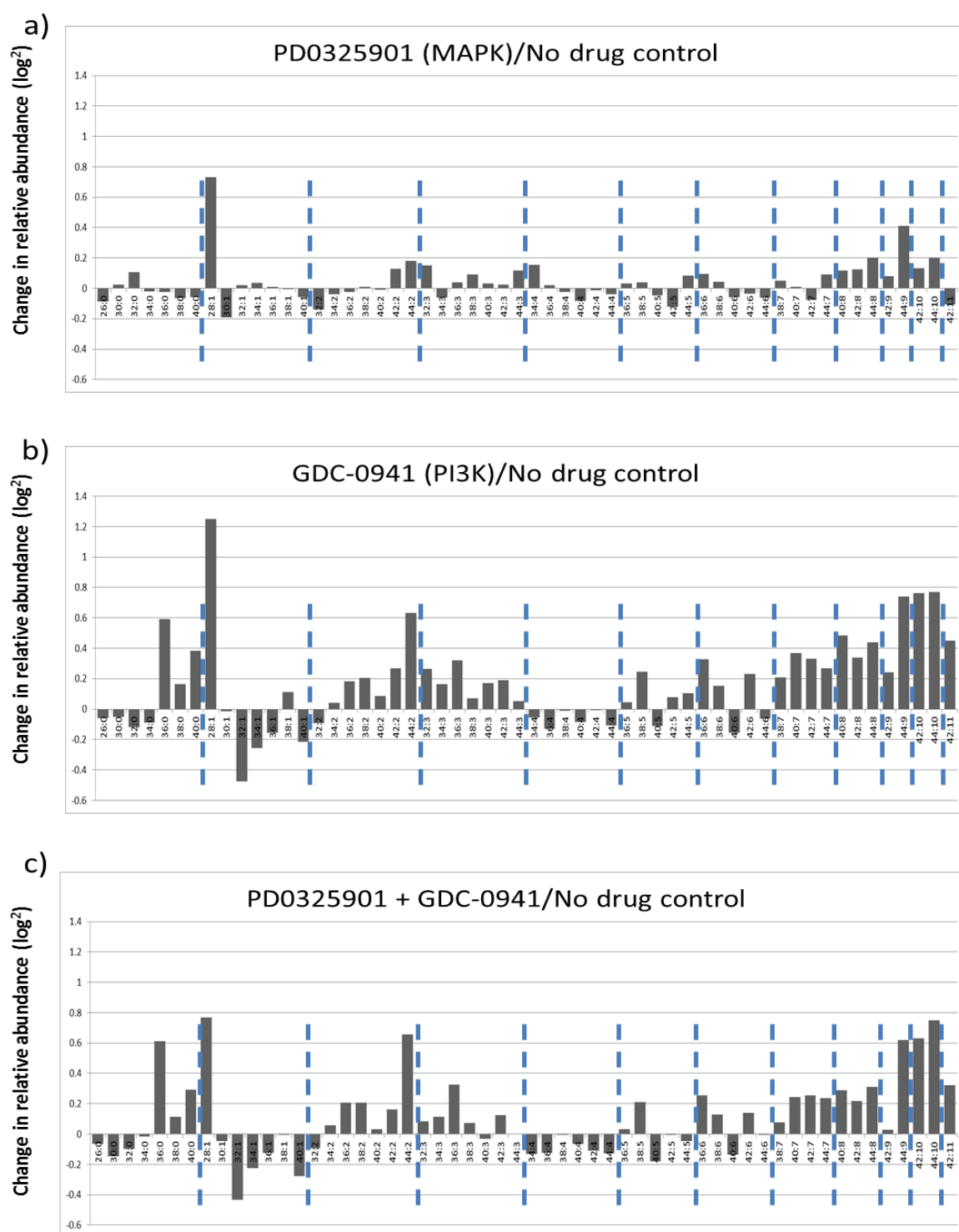


Figure 3-17: Membrane lipid analysis of phosphatidylethanolamine levels when cells are treated with MAPK and PI3K inhibitors. Relative abundance of phosphatidylethanolamine and phosphatidylethanolamine species in Suit-2 cells when the cells are treated with MAPK, PI3K inhibitors either alone or together. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group (n=1).

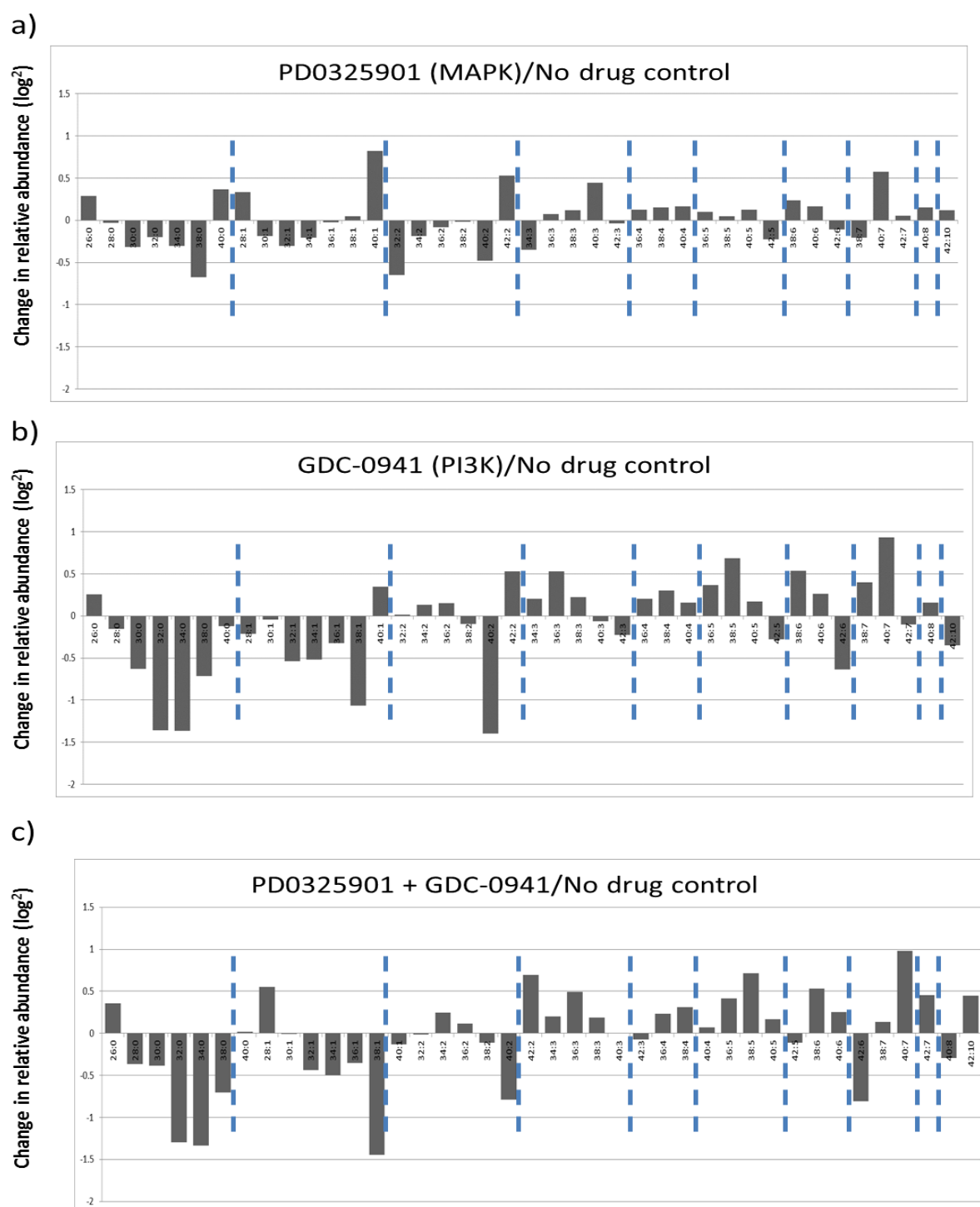


Figure 3-18: Membrane lipid analysis of phosphatidylinositol levels when cells are treated with MAPK and PI3K inhibitors. Relative abundance of phosphatidylinositol and phosphatidylinositol species in Suit-2 cells when the cells are treated with MAPK/PI3K inhibitors either alone or together. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group (n=1).

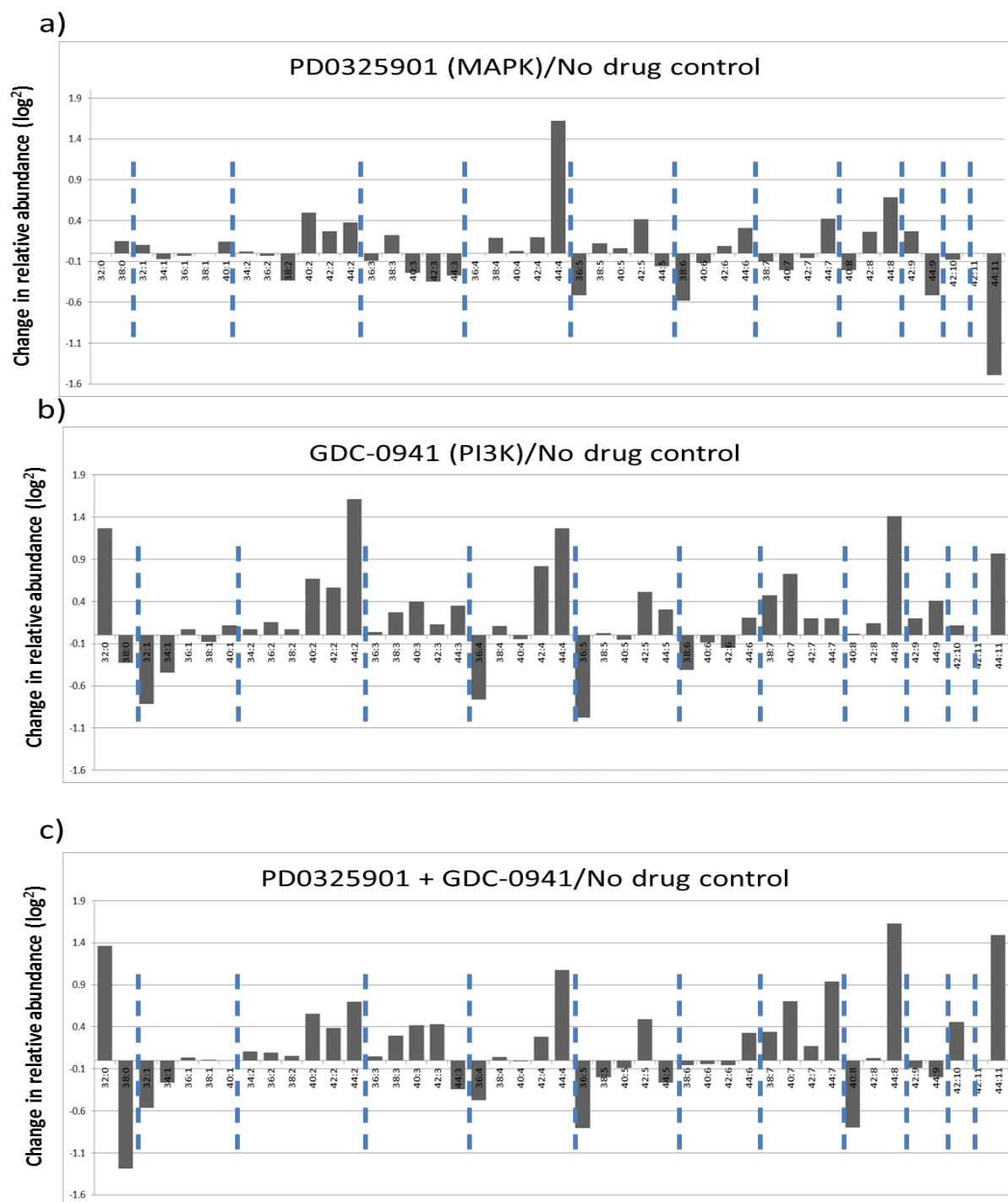


Figure 3-19: Membrane lipid analysis of phosphatidylserine levels when cells are treated with MAPK and PI3K inhibitors. Relative abundance of phosphatidylserine and phosphatidylserine species in Suit-2 cells when the cells are treated with MAPK/PI3K inhibitors either alone or together. The dotted lines represent groups with the same unsaturation and the chain length increases left to right as you move across the group (n=1).

### 3.5 SUMMARY

My hypothesis: *no other Ras isoforms apart from K-Ras are active or functional in pancreatic cancer cells* can be rejected as I demonstrated evidence of N-Ras activity via Raf and depletion of N-Ras changed the oxidative vs non oxidative growth profile of pancreatic cancer cells when depleted in conjunction with K-Ras. Additionally, I demonstrated evidence suggesting that the cytokines IL-8 and IL-12 display changes in the levels secreted when N-Ras is depleted. These changes appear to be different to those observed when the cells were compared to controls or cells where K-Ras was depleted. N-Ras seems to have little effect on the phospholipid profiles of the cells, at least in comparison with the impact of K-Ras in these cells. Indicative of the complexity of the roles of the different Ras isoforms, acting together and independently although further investigation will be required to fully understand the impact of N-Ras on phospholipid profiles.



## **RESULTS 2: K-RAS MODULATION OF G2 CYCLINS**

*Hypothesis: K-Ras regulates the G2 cyclins via cyclin D.*

### **3.1 DEPLETING K-RAS BUT NOT N-RAS RESULTS IN A CHANGE IN THE EXPRESSION PATTERN OF G2 CYCLINS**

Depleting K-Ras was expected to show a decrease in cyclin D and also a decrease in cyclins A and B. (Fleming et al., 2005; Morgan, 2007) If N-Ras was involved in controlling the cell cycle then a similar result to K-Ras depletion would be expected. When we depleted K-Ras in Suit-2 cells however, little change in the G1 cyclins (D+E) and a large decrease in G2 cyclins (A or B) was observed (Figure 3-1. Depletion of N-Ras did not show a similar pattern and it appears in fact, to have little effect on the levels of the cyclins at all. Both K-Ras and N-Ras were depleted (Figure 3-1b) indicating that the result was not due to inefficiencies in the siRNA treatment.

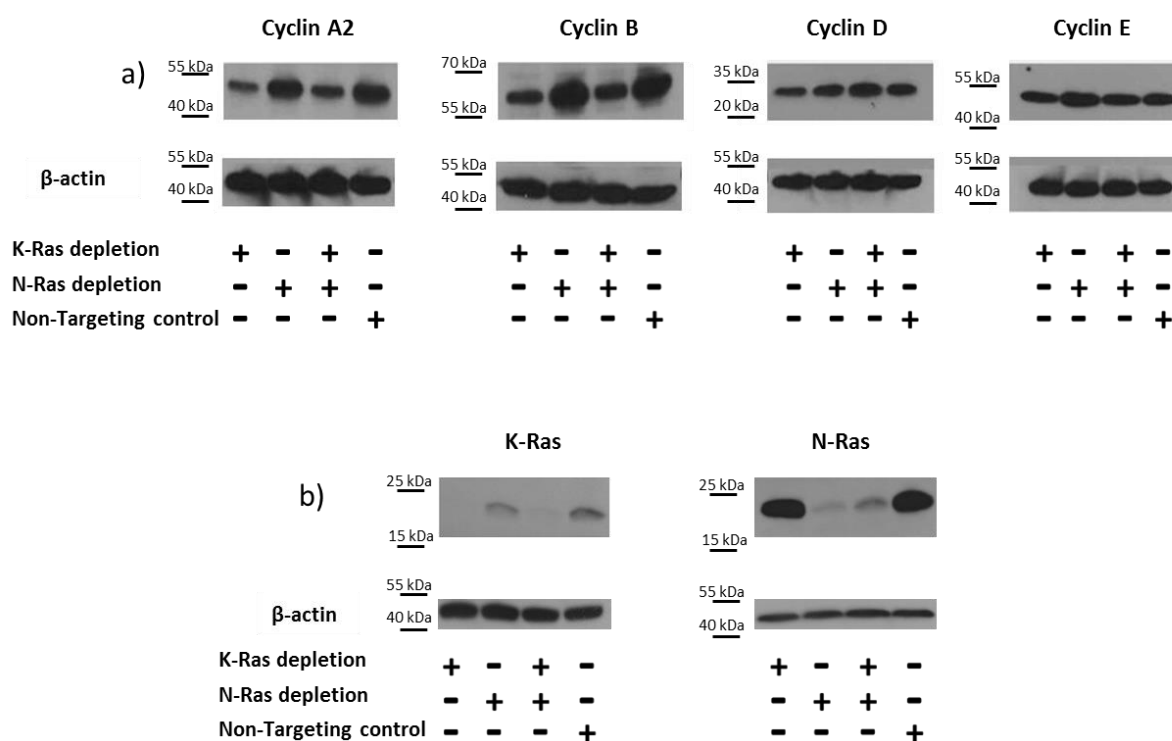
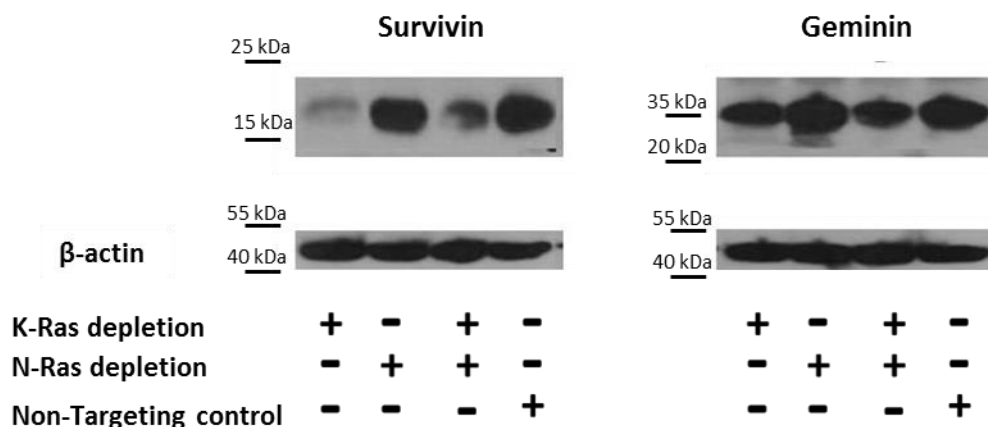


Figure 3-1: Cyclin levels when K or N-Ras were depleted in Suit-2 cells. a) Representative western blot showing the levels of cyclins when Suit-2 cells were treated with K-Ras or N-Ras siRNA (n=3). b) Western blots demonstrating the depletion of K-Ras and N-Ras (n=3).

### 3.2 EFFECT THAT DEPLETION OF K-RAS AND N-RAS HAS ON OTHER TARGETS OF THE APC/C COMPLEX

The APC/c complex is known to be responsible for targeting the G2 cyclins to the proteasome (Peters, 2002). Therefore, it is possible that K-Ras is regulating the APC/c complex, and evidence to support this would be seen if other APC/c targets show similar patterns of response to the depletion of the different Ras isoforms. Two other targets survivin and geminin were tested to see how K-Ras and N-Ras depletion effected their levels. N-Ras has little to no effect on the levels of these proteins in a similar manner to that observed with the G2 cyclins. K-Ras depletion, however, resulted

in a loss of both survivin and geminin in a similar manner to that observed for the G2 cyclins (Figure 3-2 and Figure 3-1). Therefore, it appears that K-Ras is affecting levels of G2 cyclins and this may be caused through its effects on the APC/c complex.



**Figure 3-2: Levels of proteins targeted by the APC/c after K or N-Ras are depleted. Representative western blots showing the levels of two other targets of the APC/c complex when K-Ras and N-Ras were depleted using siRNA (n=3).**

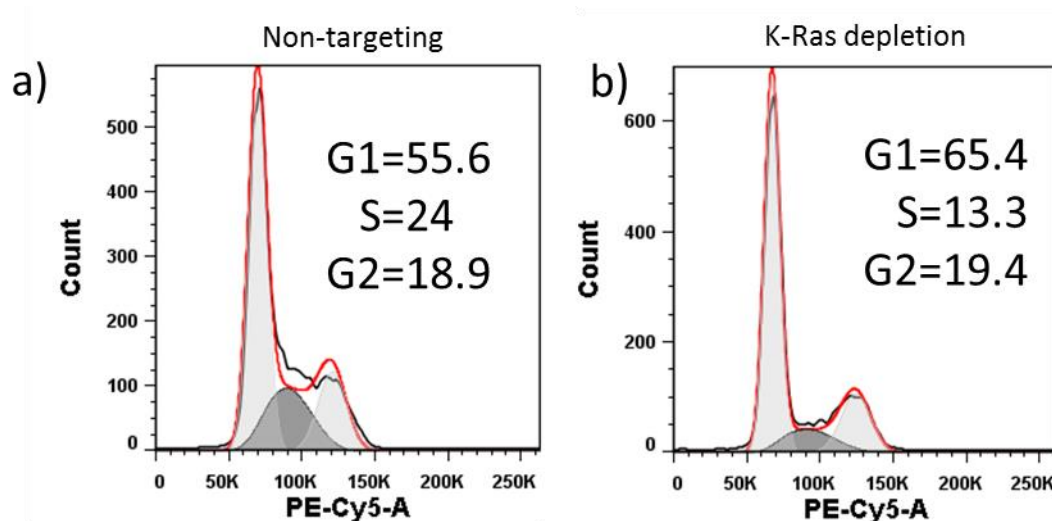
### 3.3 FACS ANALYSIS OF SUIT-2 CELLS WHEN K-RAS IS DEPLETED.

#### 3.3.1 PROPIDIUM IODIDE ANALYSIS

One possible explanation for the results observed with the G2 cyclins and other APC/c targets would be that G2 cells are being lost. If the G2 cell population was significantly decreased then a drop in these different G2 proteins would be expected. Therefore, FACS analysis was done to see if there was a reduction in G2 cells.

The FACS analysis showed that when K-Ras was depleted there was little change in the G2 peak, but a reduction in S-phase was observed (Figure 3-3). This indicates that K-

Ras depletion was not causing G2 cells to be lost in greater numbers and therefore is unlikely to explain the change in G2 cyclin levels (Figure 3-1).

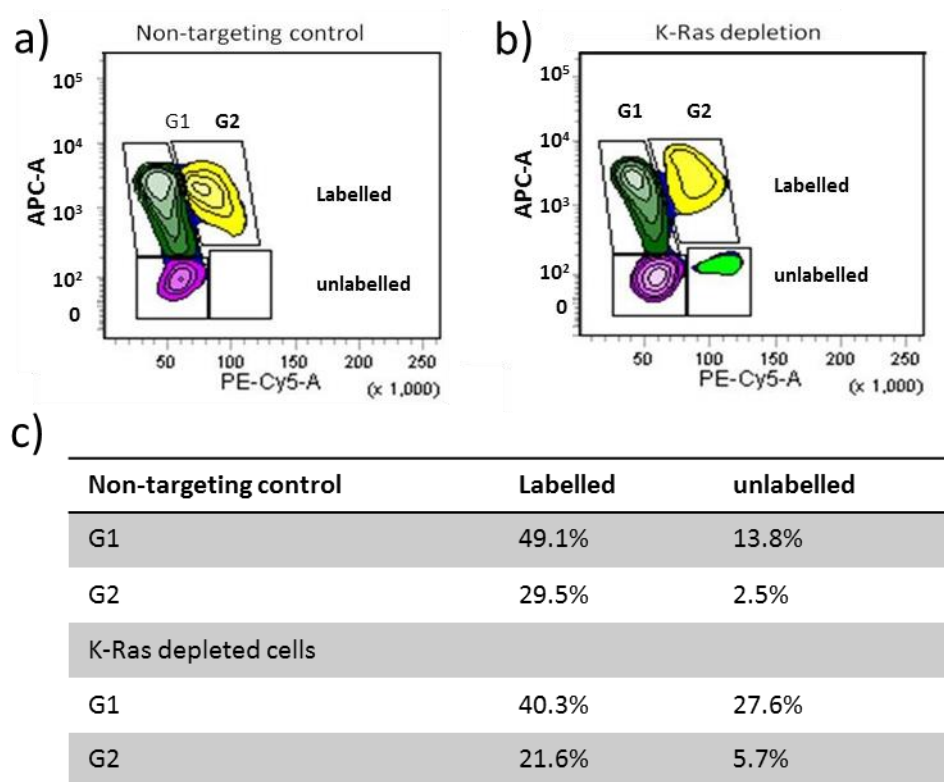


**Figure 3-3: FACS analysis following K-Ras depletion. Representative FACS analysis of the cell cycle using PI staining demonstrating a decrease in S-phase but little change in the G2 cell population. a) Non-targeting siRNA treated cells (n=3); b) K-Ras depleted Suit-2 cells (n=3)**

### 3.3.2 EDU FACS ANALYSIS

Further analysis of the cell cycle was done in Suit-2 cells using 5-ethynyl-2'-deoxyuridine (EdU) to examine if cells continue to progress through S-phase and if the number of cells in G2 is changing after K-Ras depletion. EdU incorporates in DNA when it is being replicated in S-phase. Any cell that has passed through S-phase after the addition of EdU will be labelled. Using this we can determine the cell cycle fate of cells that pass through S-phase. K-Ras depletion results in an increase in the proportion of cells that are unlabelled G2 cell population (Figure 3-4). In order for these cells to be unlabelled in G2 they must have been present in G2 since labelling began. This

demonstrates that K-Ras depletion far from forcing cells out of G2, actually causes a relative increase in a subpopulation of cells which started in G2 and have not left.



**Figure 3-4: EdU cell cycle analysis of K-Ras depleted Suit-2 cells. An example of EdU analysis of the cell cycle showing that the proportion of unlabelled G2 cells increases following the depletion of K-Ras. a) Non-targeting control treated cells, b) K-Ras depleted cells, c) Table showing relative % distribution of cells in cell cycle phases.**

The effect of the period of exposure to EdU was analysed. When K-Ras was depleted, the unlabelled population of G2 cells relative to the total number of G2 cells decreased with increasing time of EdU exposure; suggesting cells were slowly moving from G2 into G1 (Figure 3-5). When there was no K-Ras depletion however, there was a constant proportion of cells in G2 that did not change; suggesting that cells are entering and leaving G2 at a similar rate; the constant level of unlabelled cells represent a non-

cycling population. The level of unlabelled G2 cells after K-Ras depletion decreases towards the constant level of non-cycling cells seen with no K-Ras depletion. When examining the number of unlabelled cells in G2 as a proportion of the total number of unlabelled cells, it is expected that a decrease would be seen as the cells cycle from G2 to G1 (being replaced by labelled cells in G2). This was seen with cells treated with control siRNA but not in cells with K-Ras depletion (Figure 3-5). Suggesting that after K-Ras depletion cells must be leaving G1 at approximately three times the rate that they are leaving G2 (1 cell leaving G2 + 2 entering G1 due to cell division = change of 3 cells in total). This data suggests that K-Ras depletion is in fact slowing the loss of cells from G2 rather than promoting it. Together, the FACS data shown suggests that loss of G2 cells does not explain the decrease in G2 cyclins when K-Ras is depleted and therefore, K-Ras must promote their upregulation by another manner.

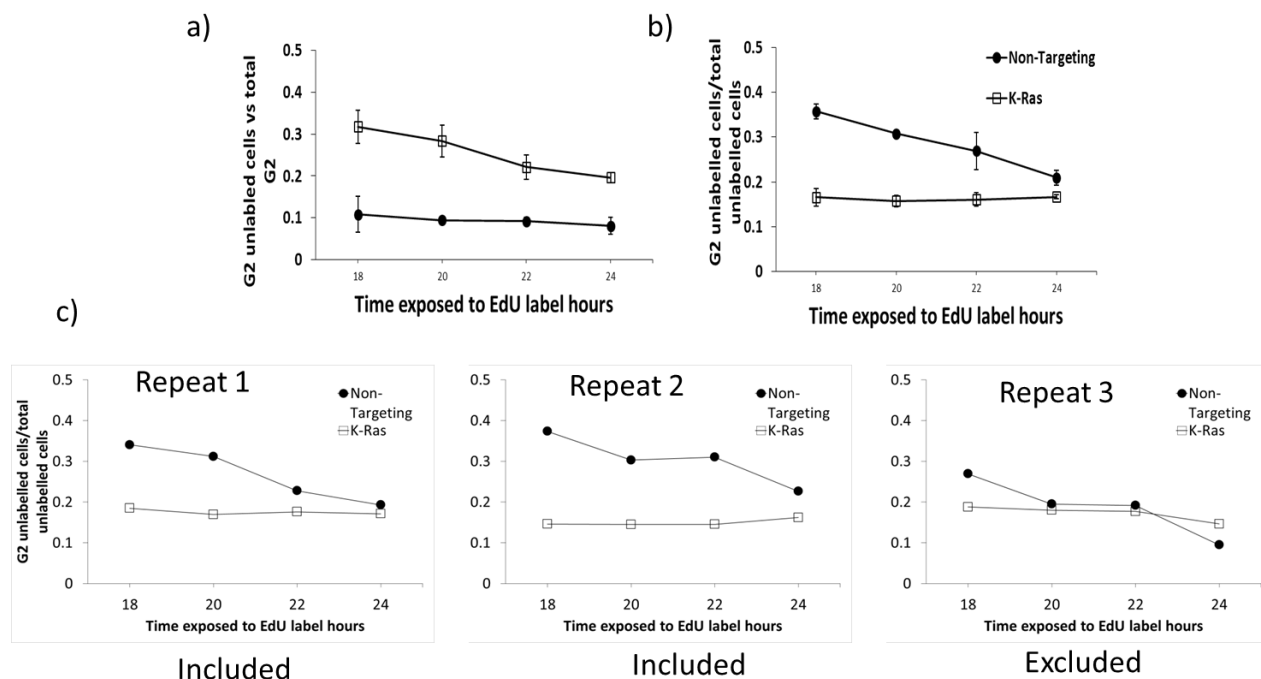


Figure 3-5: Analysis of EdU labelling over time. a) Comparison of changes to the proportion of G2 unlabelled cells vs total G2 over increasing periods of exposure to Edu when K-Ras is depleted (n=2). b) Comparison of the change in the proportion of G2 unlabelled cells vs total number of unlabelled cells over increasing periods of exposure to Edu when K-Ras is depleted (n=2). c) A 3<sup>rd</sup> repeat has been done and gave the same trend but due to lack of synchronisation the time points were too far offset to allow data to be included in an average for each point, % of unlabelled in G2 for repeats 1, 2 and 3 are shown, notice that 22 hour time point for 1 and 2 are equivalent to 18 hours for 3.

### 3.4 IS MAPK OR PI3K/AKT SIGNALLING RESPONSIBLE FOR DEPLETION OF CYCLINS?

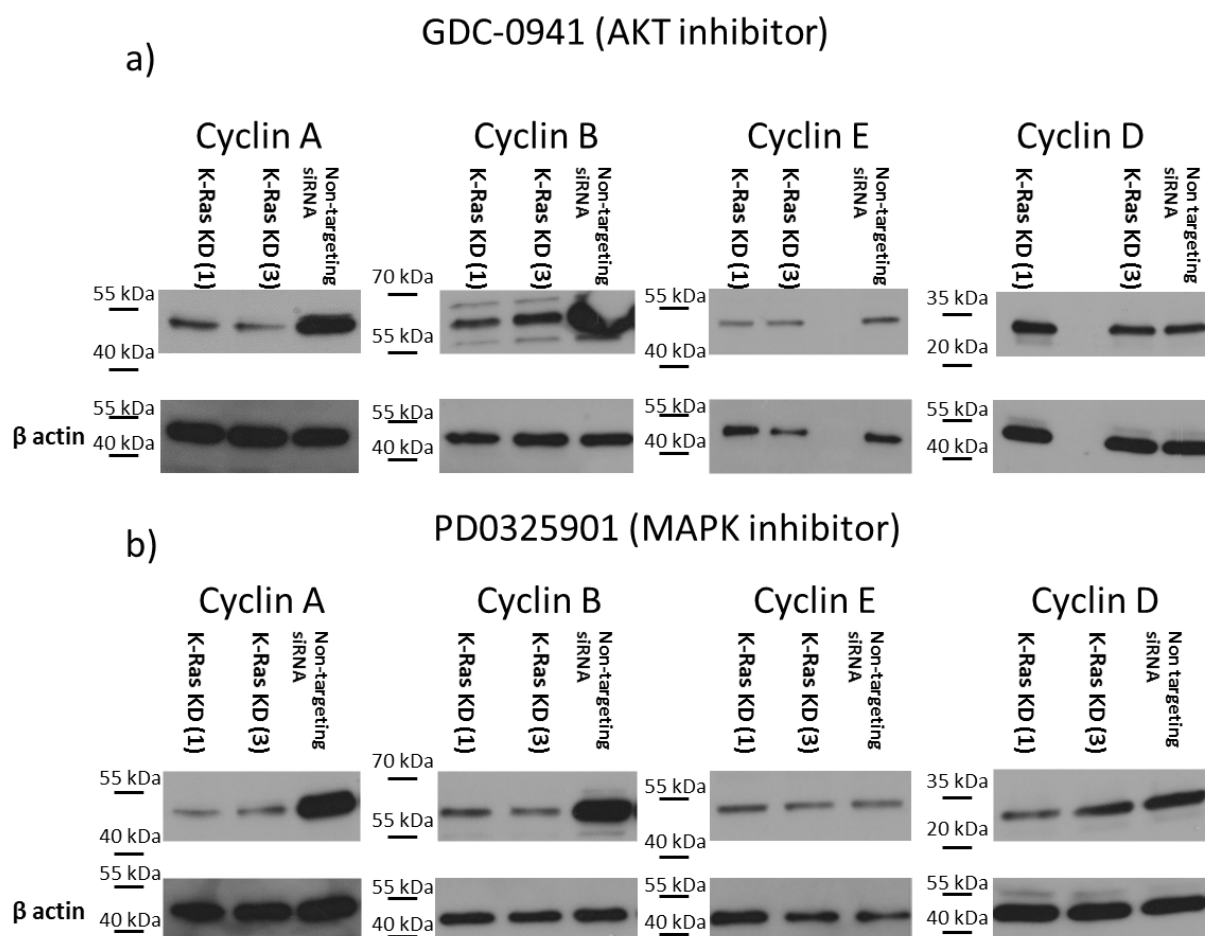
K-Ras functions through many signalling pathways, but two of the most common ones are the MAPK pathway and the PI3K/Akt pathways. In order to determine if either one of these pathways were involved in controlling the levels of G2 cyclins I used the MEK inhibitor PD0325901 and the PI3K inhibitor GDC-0941. Cells that were treated with K-

Ras siRNA or control siRNA were given these inhibitors 24 hours post siRNA treatment and were then left for a further 24 hours before harvesting. If K-Ras is controlling the cells through either of these pathways, inhibition of the pathway would cause control and K-Ras siRNA treated cells to look similar. Treatment with control siRNA followed by inhibitor treatment did not cause protein levels of the G2 cyclins to look similar to when they are treated with K-Ras siRNA (Figure 3-6 compared to Figure 3-1). Treatment with these inhibitors did not appear to demonstrate an effect on cyclin D or cyclin E either. Cyclin D and cyclin E showed little change in protein levels when K-Ras was depleted (Figure 3-1) and the addition of the inhibitors had little or no effect on the expression pattern of the protein (Figure 3-6).

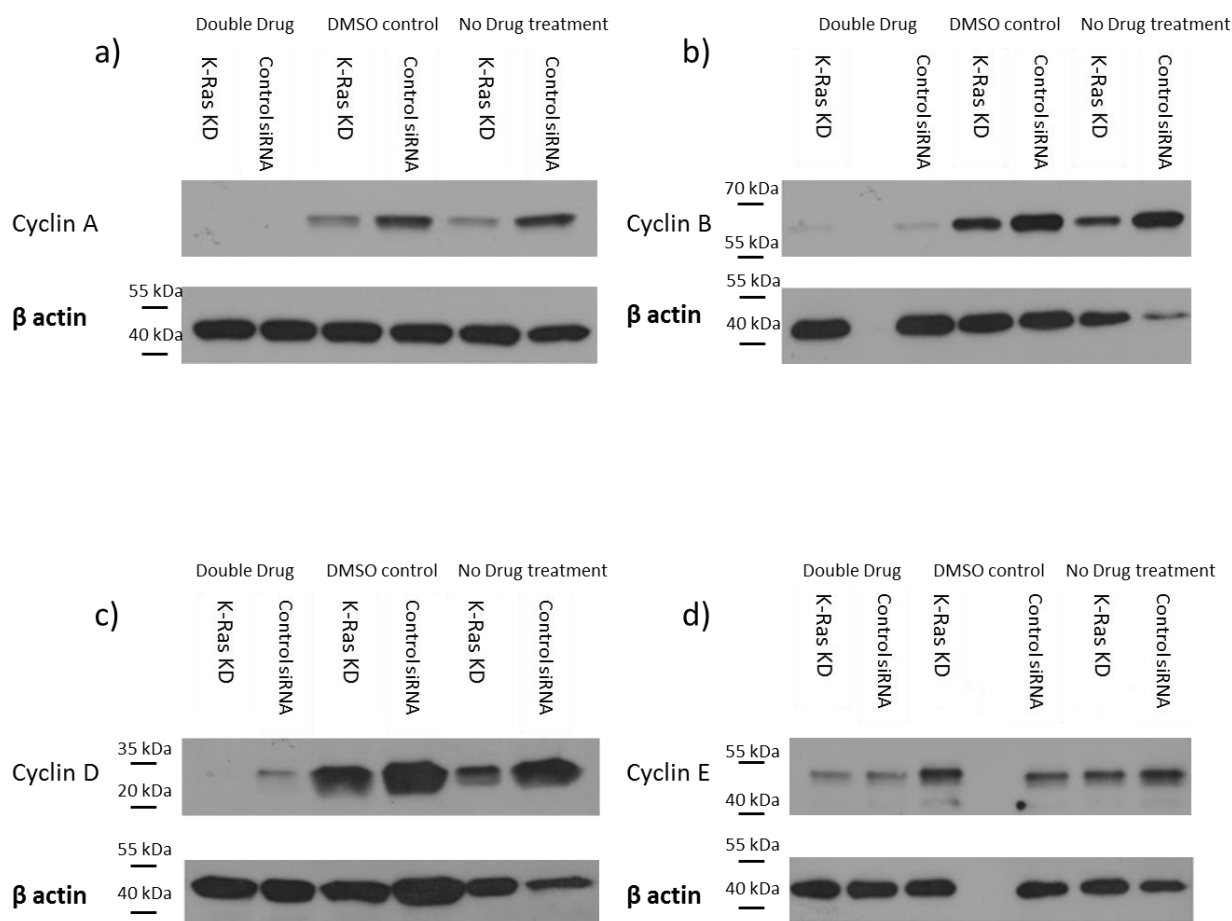
Inhibition of MAPK or Akt signalling alone does not appear to be effective in eliminating the difference between cells treated with K-Ras siRNA and control siRNA. Therefore, I repeated the experiment and added both drugs concurrently 24 hours after siRNA treatment. This was done to examine if this inhibition of both pathways simultaneously could eliminate the differences in G2 cyclins between the control and K-Ras depleted cells. The addition of both drugs results in no detectable level of cyclin A with either siRNA treatment and the levels of cyclin B are decreased to similar levels in both the control and K-Ras depleted cells (Figure 3-7). Therefore, it appears that loss of both MAPK and Akt signalling pathways can result in the loss of G2 cyclins. This coupled with the fact that each one individually is unable to alter the protein levels of the G2 cyclins suggests that cross talk between the pathways is able to overcome the loss of signalling from one of them alone. It is also possible that this result is not the reason for the changes observed in G2 cyclins when K-Ras is depleted, but rather, it is due to another set of signals that when lost may result in the loss of G2 cyclins



independently of K-Ras. Treatment of Suit-2 with both drugs also results in decreased levels of G1 cyclins and the overall pattern was the same with either siRNA (Figure 3-7).



**Figure 3-6: Effect of MAPK and Akt inhibitors on cyclin levels when K-Ras is depleted. Representative western blot showing changes in cyclins levels when (a) Akt (GDC-0941) and (b) MAPK (PD0325901) are inhibited and cells are treated with K-Ras or control siRNA (n=3).**



**Figure 3-7: Effect of combined treatment of MAPK and Akt inhibitors on cyclin levels when K-Ras is depleted. Western blot showing changes in cyclins levels when Akt (GDC-0941) and MAPK (PD0325901) are inhibited with or without K-Ras depletion. The addition of both drugs results in almost complete loss of both cyclin A and cyclin B. Cyclin D and E also have decreased levels. These effects are independent of K-Ras depletion. a) cyclin A blot, b) cyclin B blot, c) cyclin D blot, d) cyclin E blot (n=3).**

### 3.5 FACS TIME COURSE COMPARING K-RAS AND CYCLIN D KNOCKDOWNS

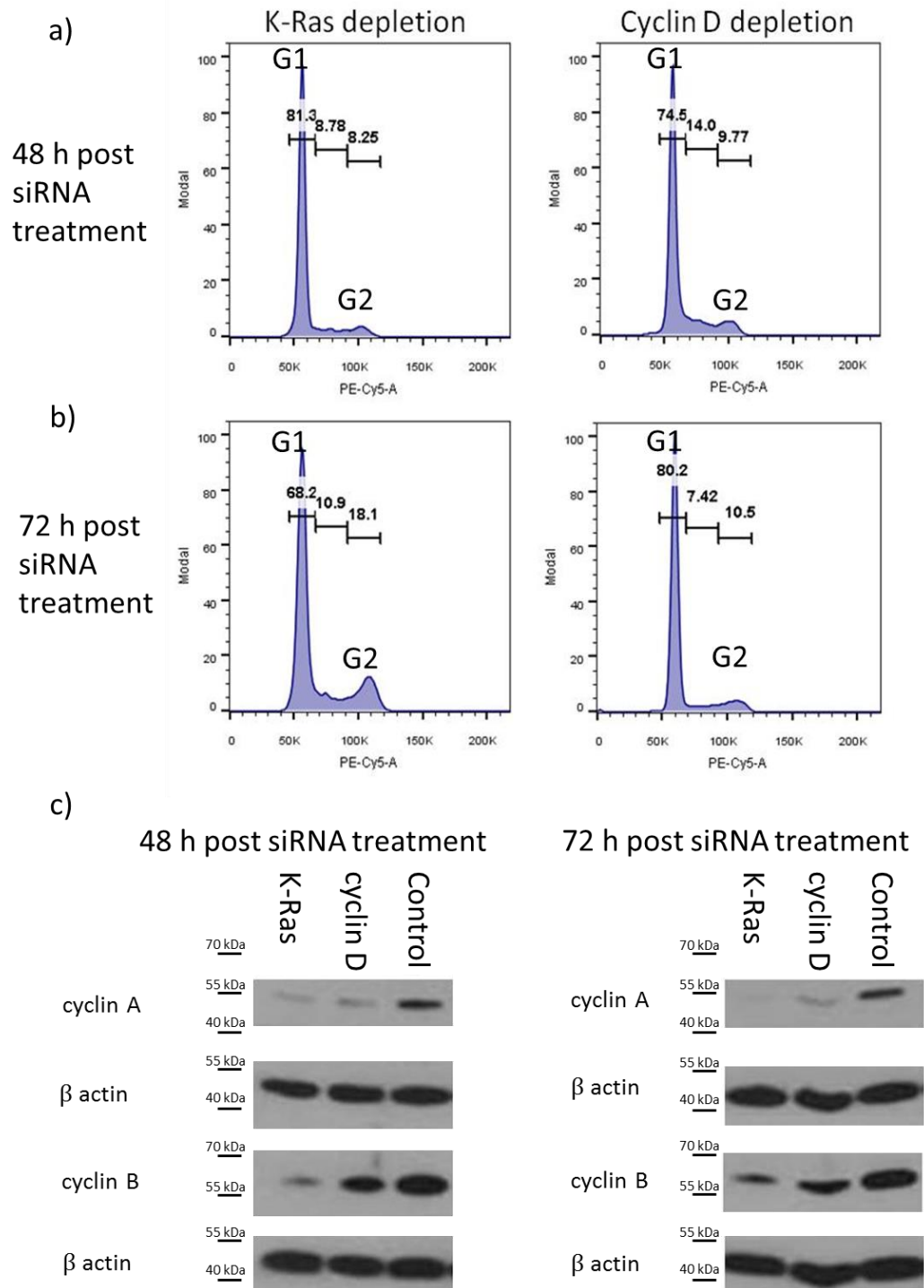
K-Ras is thought to function through cyclin D to push cells through S-phase into G2. My inhibitor data suggests that this may not be the case in Suit-2 cells. If K-Ras functions through cyclin D it would be expected to show a similar pattern of decreasing G2 cells when either cyclin D or K-Ras is depleted. In a single experiment using Suit-2 cells, when I depleted cyclin D and measured the cell cycle at both 48 hours and 72 hours post depletion, there were approximately 10% G2 cells at both time points. When I looked at K-Ras depletion it can be seen that there is approximately 10% G2 cells after 48 hours but it had risen to 18% after 72 hours (Figure 3-8a+b). Western blot analysis of the G2 cyclins shows that they are decreased after both treatments and comparison between K-Ras and cyclin D depletion would suggest that the G2 cyclins are lower after loss of K-Ras. Therefore, this data is further evidence suggesting that K-Ras is unlikely to be acting exclusively through cyclin D in Suit-2 cells.

### 3.6 POTENTIAL INTERMEDIATES BETWEEN K-RAS AND THE G2 CYCLINS

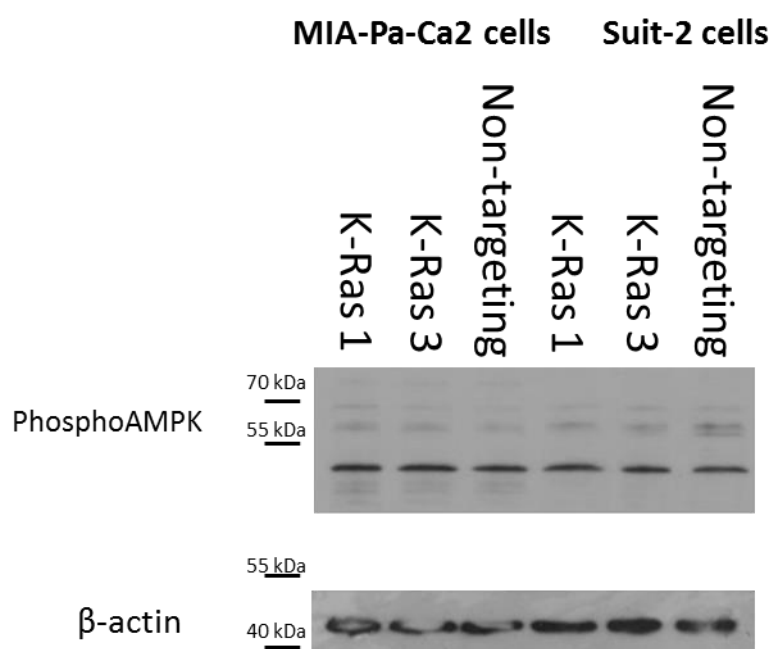
#### 3.6.1 AMPK

K-Ras does not appear to be interacting with the G2 cyclins exclusively via cyclin D, therefore, there is likely to be some other mechanism to facilitate its control of the G2 cyclins. One possibility could be that K-Ras is regulating AMPK. Phosphorylation of AMPK results in its activation, therefore if K-Ras is activating AMPK I would expect to see a decrease in phosphoAMPK when it is depleted. This would also be an alternative explanation linking K-Ras and changes in lipid profile (via mitotic exit checkpoint regulation). K-Ras was depleted and phosphoAMPK levels were examined in both Suit-2 and MIA PaCa2 cells. The depletion of K-Ras using either K-Ras siRNA 1 or 3

did not change the levels of phosphoAMPK. This indicates that AMPK is unlikely to be the intermediate as K-Ras does not appear to alter its activation, and it would therefore be unable to mediate a change in G2 cyclin levels in response to the gain or loss of K-Ras.



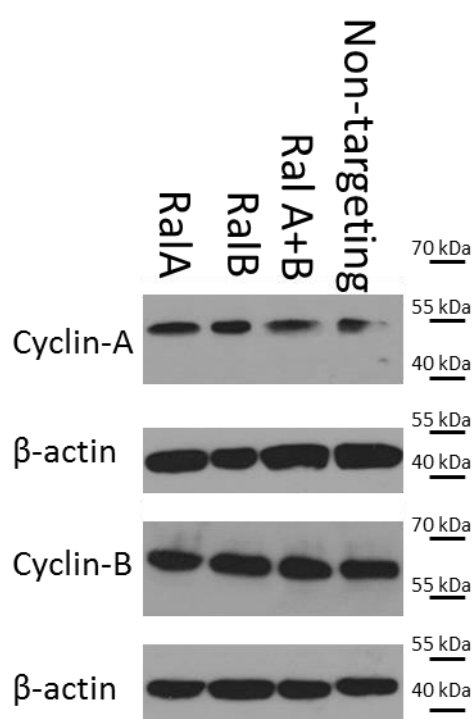
**Figure 3-8: Comparison of the effects of both K-Ras and cyclin D depletion on the cell cycle and G2 cyclins. a) PI plot of cell cycle 48 h b) or 72 h after K-Ras or cyclin D depletion showing how they affect the presence of cells in G1 or G2. c) Western blot showing the levels of G2 cyclins 48 h or 72 h post K-Ras or cyclin D depletion (n=1).**



**Figure 3-9: PhosphoAMPK and how its levels change in response to K-Ras depletion. PhosphoAMPK levels in response to K-Ras depletion were looked at in Suit-2 and MIA PaCa2 cells (n=1).**

### 3.6.2 RAL A AND RAL B

It has previously been shown that Rals are able to alter the level of survivin in response to K-Ras. (Tecleab and Sebti, 2013) Therefore, I wished to investigate if this was also the cause of the changes in G2 cyclins because previously I had demonstrated that depletion of K-Ras, resulted in a similar decrease in both survivin (Figure 3-2) and the G2 cyclins (Figure 3-1). RalA and RalB were depleted independently and in conjunction with each other with the effect on the G2 cyclins being examined. The loss of either Ral, or both together based on a single experiment had little effect on the level of the G2 proteins (Figure 3-10). Therefore this suggests that K-Ras is not controlling the levels of G2 cyclins via the RalGDS pathway.



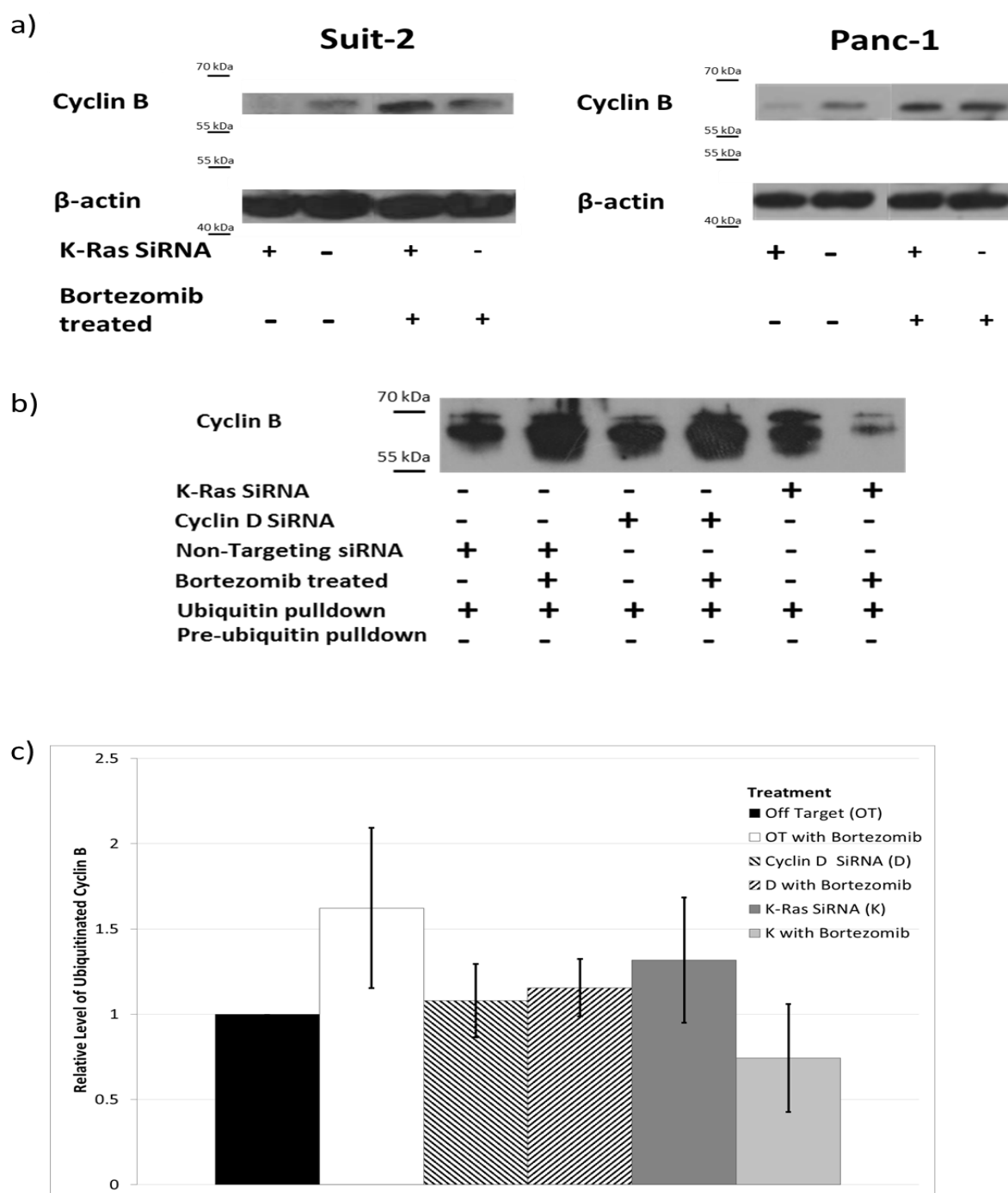
**Figure 3-10: Depletion of Rals and its effect the levels of G2 cyclins. Ral A or B were depleted either independently or in conjunction with each other and their effect on G2 cyclins was measured (n=1).**

### 3.7 BORTEZOMIB TREATMENT OF K-RAS DEPLETED CELLS

Bortezomib treatment results in the inhibition of the proteasome and therefore any proteins targeted to the proteasome via the APC/c complex will not be degraded. K-Ras depleted cells were treated with bortezomib and the level of cyclin B was measured (Figure 3-11). The addition of K-Ras siRNA degradation of cyclin B as previously demonstrated (Figure 3-1). The addition of bortezomib appears to counter this effect because when K-Ras was depleted, and the cells were treated with bortezomib, the levels of cyclin B do not decrease.

To further show this I repeated the treatment as above, but used an ubiquitin pull-down kit to specifically isolate only proteins that have been tagged by ubiquitin. When I did the western blot of this, a similar recovery of G2 cyclins following bortezomib treatment was expected. However, bortezomib with K-Ras depletion appears to cause a decrease in ubiquitinated cyclin B compared to K-Ras depletion alone. Cyclin D depletion followed by bortezomib treatment shows the expected recovery of cyclin B protein (Figure 3-11). If K-Ras increased G2 cyclins by inhibiting the APC/c complex the proteasome inhibitor would result in increased cyclin B levels. The decrease in total cyclin B (Figure 3-11a) suggests that whatever the mechanism by which K-Ras is controlling the levels of G2 cyclins it involves degradation of an intermediate protein via the proteasome. This is further evidence that K-Ras is not functioning through cyclin D because the bortezomib treatment effects the depletion of either K-Ras or cyclin D differently (Figure 3-11).

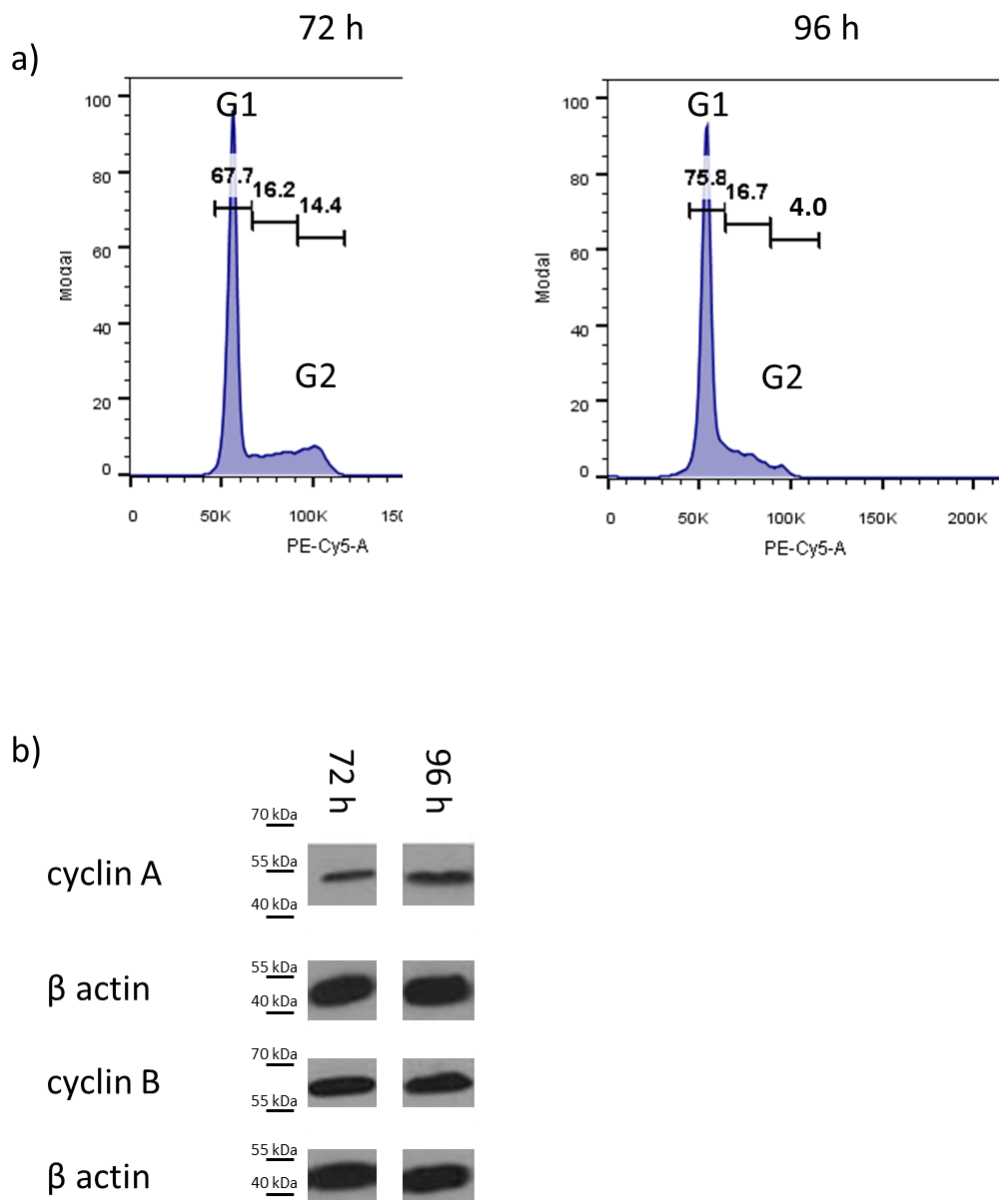




**Figure 3-11: K-Ras dependent increase in G2 cyclin level is not the result of blocking proteolysis of ubiquitinated cyclins** a) Representative western blots showing the effect of K-Ras depletion on total cyclin B in and how bortezomib treatment alters the effect Suit-2 cells (n=3) Panc-1(n=1) b) Representative western blot showing the effect of bortezomib and siRNA in combination Suit-2 cells (n=3). c) Densitometry scan of westerns represented in b (n=3). K-Ras depletion does not significantly decrease ubiquitinated cyclin B. K-Ras depletion in the presence of bortezomib decreases the level of ubiquitinated cyclin B.

### 3.8 **FACS ANALYSIS AND WESTERN BLOTS TO DETERMINE IF G2 CYCLINS ARE PRESENT IN G1**

If K-Ras is not effecting the levels of G2 cyclins via the APC/c complex it is possible that the G2 cyclins are no longer limited to G2. To test this I looked at Suit-2 cells as they become confluent. When they are growing less/slower, more cells will be in found in G1/0 than in G2 compared to when they are in an exponential growth phase. (Kuppers et al., 2010) Therefore Suit-2 cells were grown to confluence to decrease the number of cells that were in G2. After 96 hours it was found that most of the cells were in G1 (75.8% at 96 hours compared to 67.7% after 72 hours) with the G2 peak having decreased to just 4.0% of the total cell population from 14.4% at 72 hours (Figure 3-12). When the G2 cyclins were examined by western blot there was little change. This suggests in conjunction with Figure 3-8, that not only is K-Ras affecting the cells in a manner that is not the same as the classically considered cyclin D pathway, but that the G2 cyclins are possibly produced in cells outside of G2.



**Figure 3-12: Analysis to determine the presence of G2 cyclins in cells predominately in G1 phase cells. a) PI plot of cells at 72 h and 96 h (n=1) b) Western blot probed for cyclin A and cyclin B to examine how the G2 cyclin levels were affected when most of the cells were in G1 (n=1).**

### 3.9 TRANSCRIPTIONAL ANALYSIS OF G2 CYCLINS WHEN K-RAS IS DEPLETED

G2 cyclins do not appear to be restricted to G2 or controlled via the APC/c complex by K-Ras. Therefore I used Quantitative Reverse Transcriptase Real Time PCR (qRT-PCR) to assess if the regulation of the G2 cyclins is at the transcriptional level. Depletion of K-Ras results in a significant decrease in cyclin B transcript levels and a much smaller decrease in cyclin A (Figure 3-13). The loss of cyclin D also results in a decrease of the G2 cyclins. K-Ras depletion causes a much greater loss of cyclin B transcripts than cyclin D depletion. This provides further evidence that K-Ras is controlling cyclin B in an independent manner to cyclin D, and that the control of cyclin B at least is via transcriptional regulation. The low level of the reduction in cyclin A transcripts (roughly equivalent to that seen after cyclin D depletion) does not eliminate the possibility that it is of biological significance.

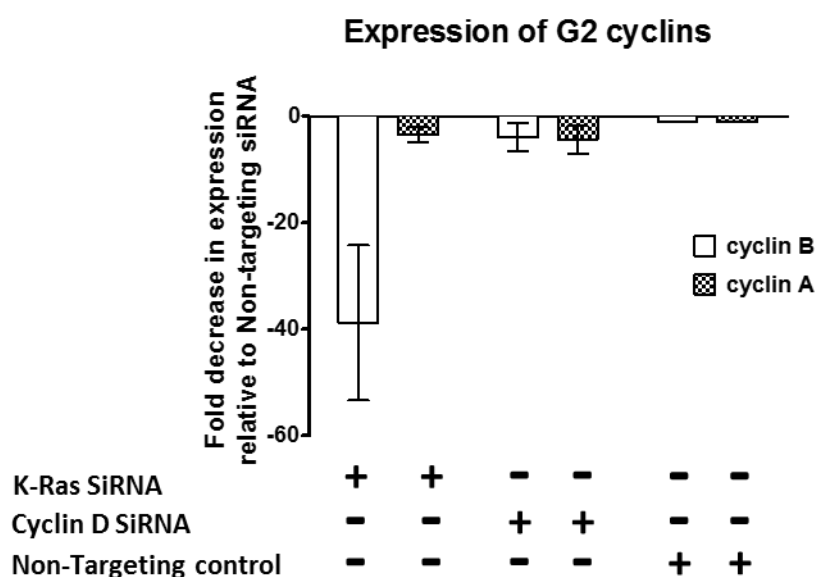


Figure 3-13: Effect of depleting K-Ras (cyclin A n=4, cyclin B n=5) or cyclin D (cyclin A n=3, cyclin B n=4) on G2 cyclin transcript levels. Graph showing the decrease in G2 cyclin levels following K-Ras or cyclin D depletion.

### 3.10 EFFECT OF BORTEZOMIB ON THE LEVELS OF G2 CYCLIN TRANSCRIPTS

#### WHEN K-RAS IS DEPLETED

There is evidence that the depletion of K-Ras results in a decrease in G2 cyclin transcripts (Figure 3-13). When the cells were also treated with bortezomib, an increase in cyclin B transcript levels and a decrease in the transcription levels of cyclin A (Figure 3-14) was observed, suggesting that bortezomib can recover some of the loss in cyclin B transcription following K-Ras depletion. The treatment with bortezomib alone with control siRNA demonstrated an increase in the transcription levels of both cyclin A and B (Figure 3-14).

## Expression of G2 cyclins with or without the presence bortezomib

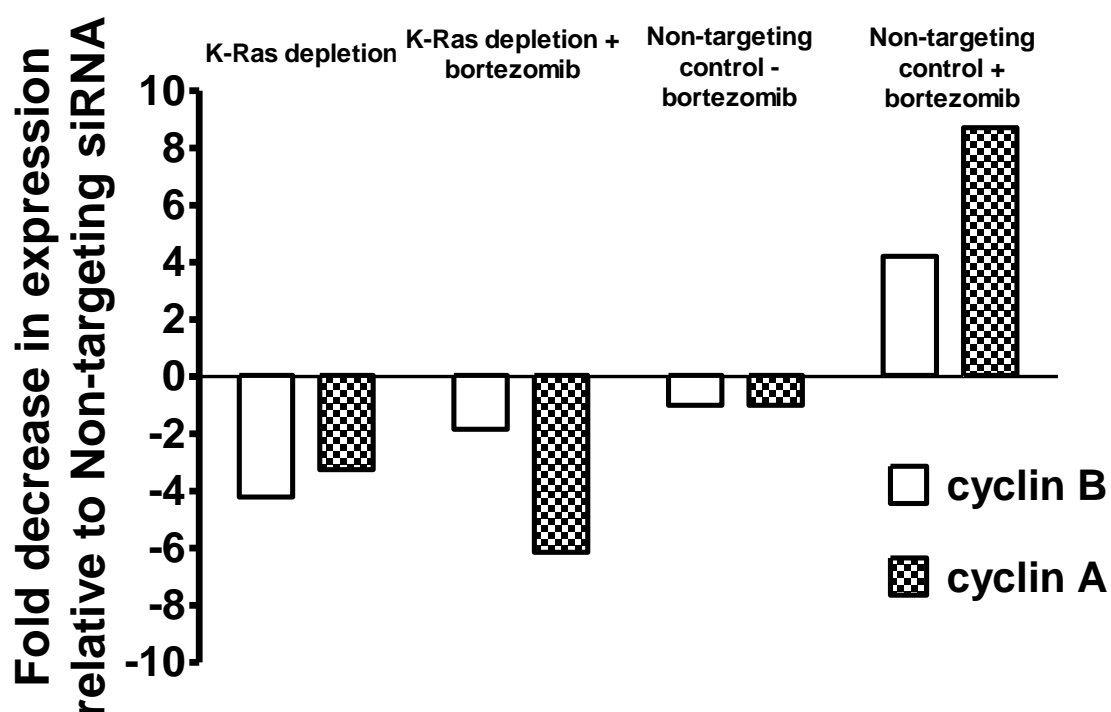


Figure 3-14: Effects on G2 cyclins when Suit-2 cells are treated with K-Ras siRNA and bortezomib. Graph showing the effect when Suit-2 cells were treated with K-Ras siRNA or Non-targeting siRNA and then bortezomib 24 h later. (n=1).

### 3.11 SUMMARY

My hypothesis for this work was that K-Ras only regulates the G2 cyclins via cyclin D in a cell dependent manner. This hypothesis can be rejected as depletion of K-Ras causes a depletion of G2 cyclins without a simultaneous decrease in cyclin D and does not cause a G1 arrest. K-Ras depletion in fact is demonstrated to cause a slowing of cells exiting from G2 and is likely to be causing a slowing of the cell cycle in general. The hypothesis was rejected and the APC/c was examined to determine if it was responsible for the loss of G2 cyclins. Inhibition of the proteasome was not able to recover the loss of ubiquitinated cyclin B. K-Ras depletion in the presence of Bortezomib results in a decrease in the level of ubiquitinated cyclin B. The transcription of the G2 cyclins was examined and the levels of cyclin B transcript decreased more when K-Ras was depleted, than when cyclin D was depleted. Therefore, I have provided evidence that K-Ras is able to modulate the levels of G2 cyclins in a manner that is independent of the cell cycle and cyclin D. For cyclin B this regulation appears to be transcriptional.

## CHAPTER 4: DISCUSSION

### 4.1 K-RAS DEPLETION RESULTS IN VIABLE CELLS

Objectives of this thesis were to identify if Ras isoforms other than K-Ras are active in pancreatic cancer cells and if they have any functions that differ to K-Ras, then to examine how active Ras isoforms modulate cyclin levels in PDAC cell lines, and identify if cyclin regulation extends beyond the classical paradigm described for cyclin D. I had two hypothesis based on the literature; that *no other Ras isoforms are active or functional in pancreatic cancer cells* and that *K-Ras only regulates the G2 cyclins via cyclin D in a cell cycle dependent manner*.

The objectives of this thesis relate to K-Ras dependency, so I first set out to confirm and further elucidate the effects that K-Ras has on specific pancreatic cancer cells. K-Ras depletion using siRNA (as demonstrated by western blot) in Suit-2, MIA PaCa 2 and Panc-1 cells was well tolerated (Figure 3-1), although it gave a relative reduction in cell growth when compared to controls. Singh *et al* have previously defined Panc-1 cells as independent of K-Ras depletion in agreement with the results that I have observed. (Singh et al., 2009) The Ras dependency state of Suit-2 cells in their analysis was in contradiction to their own hypothesis because their panel of genes for determining dependence classified Suit-2 as independent. (Singh et al., 2009) When they examined cell density following K-Ras depletion however, they concluded that they were in fact, dependent. (Singh et al., 2009) The genetic markers that were discovered by Singh *et al*, represented genes involved in epithelial differentiation and cell survival with several being involved in the apoptosis pathway. (Singh et al., 2009) I would argue that Singh *et al* correctly classified Suit-2 cells as independent based on their model and



incorrectly classified the line as dependent, due to their definition of dependency was empirically based on cell density (growth) rather than cell death. When I examined Suit-2 cells for evidence of cell death I found that the cells had similar levels of viability when treated with K-Ras or control siRNA (>90%), and that K-Ras depletion only resulted in a 6.7% increase in apoptotic cells (4.5% to 11.1%) (Figure 3-2). This therefore, suggests that the loss in cell density observed by Singh *et al*, in Suit 2 cells is likely to be due to a lack of cell growth. When the growth of Suit-2 was tracked while K-Ras was depleted, it is noticeable that there was an increase in cell growth following the restoration of K-Ras protein levels (Figure 3-3 and Figure 3-4). This provides further evidence that Suit-2 cells are dependent on K-Ras for growth rather than survival. Supporting this, cells surviving the loss of K-Ras by entering a slowed period of growth have been shown in mouse models: (Viale et al., 2014) the loss of K-Ras leaves small surviving cell (SC) populations in a slowed growth state. Once K-Ras returns to its higher, pre-depletion levels, tumours can rapidly form. (Viale et al., 2014)

#### 4.2 ACTIVE RAS ISOFORMS IN PANCREATIC CANCER CELLS

K-Ras is the only mutated isoform that is commonly seen in pancreatic cancer (Biankin et al., 2012) and therefore is commonly thought of as the being the Ras isoform that is the key controller of downstream signalling. Recently there has been some evidence to show that the other Ras isoforms are also present in pancreatic cancer cells, (Omerovic et al., 2008) as well as some basic evidence for a possible function. (Young et al., 2013) Western blot analysis confirmed the presence of N-Ras in Suit-2 (Figure 3-6), Panc-1, BxPC3 cells and a cell line that had been isolated from a KPC mouse (Figure 3-7). In addition I isolated both active K-Ras and N-Ras in our human cell lines but not our

KPC mouse cell line (Figure 3-6+Figure 3-7). This demonstrates, in agreement with Young *et al*, (Young et al., 2013) that N-Ras is not just present in human pancreatic cancer cell lines but it is capable of promoting signalling at least via Raf. It also suggests that, while an invaluable model, the artificial genesis of the KPC mouse may result in it having different characteristics to, at least, some human pancreatic cancers.

Others have already demonstrated K-Ras has an important role in cell proliferation and growth as well as cell survival. (Singh et al., 2009) Since I have shown N-Ras is active in our pancreatic cancer cell lines, I wanted to determine if N-Ras was able to function (independently or with K-Ras) in controlling various different facets of cell biology. The Seahorse Bioanalyzer allows the measurement of the growth response of cells to the depletion of K or N-Ras alone or together. I examined the change in the OCR/ECAR levels (the ratio of respiratory growth to non-respiratory growth) when depleting one or both Ras isoforms in our Suit-2 cells (Figure 3-8). When I did this I saw little difference when N-Ras was depleted compared to the control and a large movement toward respiratory growth when K-Ras was depleted: which is consistent with previous reports.(Ying et al., 2012) When both were depleted simultaneously an intermediate level of OCR/ECAR was observed, it was significantly different from the K-Ras depleted cells or cells treated with control siRNA. N-Ras appears to have a function that is in opposition to that of K-Ras. The loss of N-Ras alone however, shows no significant change in OCR/ECAR, suggesting that K-Ras is the dominant isoform in determining the levels of respiratory vs non-respiratory growth.

I identified two different strains of the Panc-1 cell line that have different doubling times and when they were examined for N-Ras it was only present in the strain with the faster growth (Figure 3-9). These two different strains were then examined using the

Seahorse Bioanalyzer (Figure 3-10) and it was found that N-Ras depletion had no effect on the slower growing strain (unsurprising as it did not contain any substantial active N-Ras protein before depletion). In contrast to the Suit-2 cells, N-Ras appears to have an effect in the faster growing Panc-1 cells. Here K-Ras depletion alone changes the OCR/ECAR ratio very little compared to control treated cells. The depletion of both K and N-Ras results in a movement towards respiratory growth; noticeably similar to OCR/ECAR in the K-Ras depleted slow growing Panc-1 cell line. Although the role of mutant K-Ras in regulating oxidative phosphorylation has been described previously, (Ying et al., 2012) the role of wild type N-Ras in these cells has not previously been examined. The importance of N-Ras relative to K-Ras appears to vary according to the cell line, with K-Ras masking any effect in Suit-2, but in the normal Panc-1 cell line N-Ras appears to have an important role in the maintenance of non-respiratory growth.

Examination of a 27plex of cytokines also provided evidence that N-Ras has a functional impact on the cell. From the panel of 24 cytokines only 5 had levels that were detectable in the assay (Table 11: . Of these I observed obvious changes in two of these cytokines when comparing the depletion of N-Ras or K-Ras to each other or the control. Depleting N-Ras results in a small decrease in the levels of IL-12 compared to the control (Figure 3-11) and an increase is observed when K-Ras is depleted. IL-12 has been previously reported to be downregulated by PI3K signalling in antigen presenting cells where it is involved in stimulating the immune response.(Fukao et al., 2002) I would hypothesise that the mutant K-Ras is involved in the reduction of IL-12, which potentially may help the tumour remain hidden from the immune system. Perhaps N-Ras is functioning through another pathway (perhaps MAPK) and therefore, its loss results in a decrease in IL-12.

IL-8 has previously been shown to be a transcriptional target of the Ras signalling pathway and has been found to be important in the induction of tumour growth in cells with mutant Ras. (Yasumoto et al., 1992) The depletion of K-Ras has little effect on the level of IL-8 relative to the other cytokines, whereas the depletion of N-Ras results in an increase in the relative levels of IL-8 being secreted from the cell (Figure 3-11). The loss of both isoforms shows little change in the relative level of IL-8 when compared to the control. Based on this data, and what has been previously demonstrated, it is possible to hypothesize that N-Ras has a negative regulatory function of IL-8, and K-Ras is involved in promoting its production; possibly via the previously demonstrated transcriptional control mechanism. (Yasumoto et al., 1992)

When I looked at the lipid profiles of our cells, K-Ras appeared to be the dominant isoform in determining the profiles. On the basis that the change in profile appears somewhat similar when PI3K or K-Ras is targeted. I would hypothesise that K-Ras may be acting in this respect through the PI3K pathway rather than MAPK pathway (Figure 3-12 to Figure 3-19).

Wild type N-Ras appears to have some functions unique to it. I have also shown evidence where K-Ras appears to be the dominant Ras isoform. In these cases the loss of N-Ras has only a minor impact but that concurrent depletion of K-Ras and N-Ras results in an effect distinct from depletion of K-Ras alone ~~is depleted~~. I can conclude that N-Ras is active and has functional roles in pancreatic cancer, and therefore reject the hypothesis that *no other Ras isoforms are active or functional in pancreatic cancer cells*.

### 4.3 EFFECTS OF RAS ON THE CYCLINS

As previously mentioned, K-Ras can promote the upregulation of cyclins and so activation of key Cdk's promoting the cell cycle. Previous work has suggested that K-Ras mainly functions through cyclin D, (Bretones et al., 2014) but this thesis demonstrates little change in cyclin D levels and much larger changes in cyclins A and B (Figure 3-1). Both cyclin A and cyclin B are degraded via the APC/c and therefore, I examined the effect that depletion of K-Ras and N-Ras had on two other targets of the APC/c (survivin and geminin) (Figure 3-2). Both of these alternate targets showed results similar to those seen with cyclin A and B. This initially led me to believe that control of the levels of G2 cyclins by K-Ras was at the degradation level by control of the proteasome. This was supported by PI FACS analysis of the K-Ras depleted cells, where a G2 fraction was still observed (Figure 3-3). Others have shown that cyclin D loss causes a G1 arrest (Masamha and Benbrook, 2009) and the PI FACS analysis comparison of K-Ras depleted cells vs cyclin D, confirmed the expected cyclin D result in contrast to the result with K-Ras depletion (Figure 3-8). Therefore, the hypothesis that a loss of G2 cells explains the decrease in G2 cyclins can be rejected.

To further examine the growth patterns of Suit-2 and to elucidate how proliferation of the cells has decreased either by arrest or slowing of the cell cycle, cells entering S-phase were labelled using EdU (Figure 3-4). This experiment demonstrated that K-Ras depletion slows exit from G2, rather than promoting it. Comparison of the G2 unlabelled cells vs the total unlabelled cells suggested that K-Ras depleted cells must be leaving G1, approximately 3 times faster than they enter G2 (Figure 3-5). This would add further to my previous postulation that Suit-2 cells are dependent on K-Ras for growth but not survival. It also provides additional evidence that the changes in the G2

cyclins observed in my western blot analysis are not just side effects of a change in the cell cycle profile.

K-Ras has been shown to function through both the MAPK and PI3K pathways (De Luca et al., 2012). To determine if the change in G2 cyclins is caused via either signalling pathway I used inhibitors specific to each. Inhibition of each pathway on its own failed to demonstrate the same decrease in expression of G2 cyclins seen with K-Ras depletion (Figure 3-6). When both pathways were inhibited the G2 cyclins were decreased. This decrease was greater than the K-Ras depletion alone and double inhibitor treatment demonstrated a greater decrease in cyclin D than when K-Ras is depleted (Figure 3-7). Therefore, it is possible to suggest that G2 cyclin expression requires both pathways, and that the intercommunication between the pathways, make the inhibition of one insufficient to replicate the effects seen by K-Ras depletion. Alternatively, when both pathways are blocked loss of G2 and G1 cyclins may be due to a different mechanism than the loss of G2 cyclins with K-Ras depletion.

I have determined that the K-Ras specific effect on G2 cyclins is not solely down to the cell cycle. In addition I have shown two other targets of the APC/c (survivin and geminin) respond to the loss of K-Ras in a similar manner. I wished to investigate whether the levels of G2 cyclins were controlled by the APC/c complex and to do this I inhibited the proteasome using bortezomib. Inhibition of the proteasome will lead to an increase in ubiquitinated proteins as they are not being destroyed. (Hershko and Ciechanover, 1998) If G2 cyclins levels are controlled via the APC/c then the inhibition of the proteasome should rescue the loss of G2 cyclins following K-Ras depletion. Bortezomib treatment of cells did rescue the loss of the G2 cyclins (Figure 3-11). To confirm the direct role of an E3 ligase on G2 cyclins, (consistent with APC/c regulation

by K-Ras) I looked at the ubiquitinated G2 cyclins specifically. Following K-Ras depletion and bortezomib treatment ubiquitinated G2 cyclin levels decreased (Figure 3-11). This suggests that G2 cyclins levels are not being controlled via their targeted degradation via APC/c. However, G2 cyclin regulation must involve some form of proteasome mediated degradation, either upstream or downstream of K-Ras, because it is affected by bortezomib. Testing of cyclin D under the same conditions showed that any decrease in the level of cyclin D was recovered when the cells were also treated with bortezomib (Figure 3-11), providing further strong evidence that the K-Ras effect on G2 cyclins is not via cyclin D.

G2 cyclins, as the name suggests, are present in normal cells when they are in G2. However, when cells were concentrated in G1 (by growing them to confluence), I observed little change in the levels of the G2 cyclins (Figure 3-12). This suggests that in Suit-2 cells G2 cyclins are not just restricted to G2.

A previous study had demonstrated Ral A and Ral B to be targets for K-Ras mediated regulation of survivin in lung cancer cells. (Tecleab and Sebti, 2013) In this thesis I showed that depletion of Ral A and Ral B alone or in conjunction with each other, did not change the levels of the G2 cyclins in Suit-2. Thus it can be concluded that K-Ras is not functioning through this pathway to control the levels of the G2 cyclins.

I also found that when K-Ras was depleted, the level of cyclin B transcripts decreased several fold (cyclin A was effected much less) and concluded that K-Ras is controlling the levels of cyclin B, at least transcriptionally (Figure 3-13). This leads towards a model (Figure 4-1) whereby there is an unknown K-Ras regulated protein or proteins involved in activating transcription of G2 cyclins; this will be described below as

protein X. This protein is normally degraded via the proteasome but mutant K-Ras either directly or indirectly is involved in stabilising it. The depletion of K-Ras results in the degradation of protein X and therefore a decrease in the transcription of G2 cyclins (at least cyclin B). The addition of bortezomib stabilises protein X resulting in rescue of G2 cyclins from K-Ras depletion. Bortezomib has also been shown to stop cells entering G2. (Baiz et al., 2009) Treatment of our cells with bortezomib shows a decrease in ubiquitinated G2 cyclins (Figure 3-11). Which is likely because they remain in G1 where the APC/c mitotic complex is not active and able to ubiquitinate them. Initial data examining transcripts of cells treated with bortezomib compared to those without suggests that the treatment, results in a decrease in the number of cyclin B transcripts and an increase in cyclin A transcripts when K-Ras is depleted (Figure 3-14). Also it is notable that there appears to be a general increase in transcription of G2 cyclins when control cells are treated with bortezomib.

I have provided evidence that K-Ras controls the G2 cyclins transcriptionally through a novel, and as of yet undefined pathway which is not reliant on cyclin D. Therefore I can reject the hypothesis that I presented initially, that *K-Ras only regulates the G2 cyclins via cyclin D in a cell cycle dependent manner.*



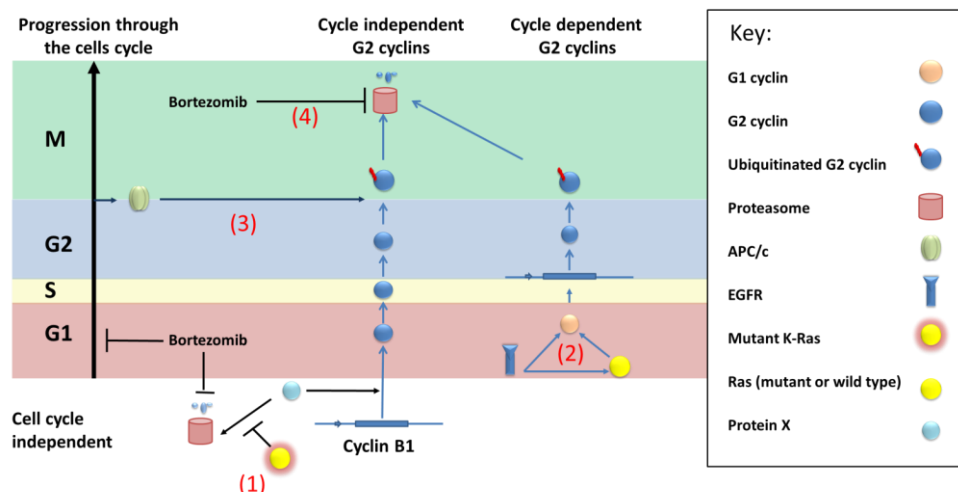


Figure 4-1: **Model suggesting a mechanism to explain non cell cycle dependent G2 cyclin levels. (1) Mutant K-Ras depletion causes a reduction in the transcription of G2 cyclins (at least B1), which is inhibited by bortezomib, suggesting the oncoprotein inhibits proteasomal degradation of a protein(s) described here as protein x. (2) Wild-type (or mutant) Ras will increase transcription of G2 cyclins via G1 cyclins in a cell cycle dependent fashion. EGFR will activate this process but can also increase G1 cyclins by directly binding to their promoters. (3) G2 cyclins will be degraded via the APC/c complex and so in the absence of mutant Ras the level of the G2 cyclins will be limited by the proportion of cells in G2. (4) Bortezomib will inhibit the proteasome causing stabilisation of ubiquitinated G2 cyclins, but will reduce the production of ubiquitinated protein by restricting entry to (and so exit from) G2.**

#### 4.4 FUTURE WORK

This work has been done mainly in the Suit-2 cell line, although I have also demonstrated functional N-Ras in other cell lines. The next step would be to examine primary cell lines to see if they show the presence of active N-Ras. If this was demonstrated to be true then I would then proceed to Luminex and Seahorse analysis to see if these cells demonstrate similar characteristics to those observed in Suit-2 cells.

I also did a preliminary study on the lipid profiles of cells with various Ras isoforms depleted. I saw that K-Ras depletions caused changes in these profiles, I would like to examine the difference between tumour and normal tissues of the same patient when the

mutation status of K-Ras is known. This can be done with our collaborators using primary cells isolated from the tumours or directly from the tissue itself.

A model has been proposed involving an undefined protein/ pathway (protein X) that is responsible for the direct control of G2 cyclins transcript levels. I would like to perform further analysis of cells treated with K-Ras +/- bortezomib vs control siRNA treated cells +/- bortezomib. Microarray analysis comparing the different conditions would help to isolate proteins that are changing levels in response to the different treatments. The identified proteins could then be depleted or overexpressed to see if the pattern of G2 cyclins were altered consistent with the model. A yeast-1-hybrid screen could be performed to identify transcription factors that bind to the G2 cyclin promoter regions; cross-referencing with the microarray data.

As well as elucidating Ras function, the identification of Protein X would provide an important target for drug discovery.

## CHAPTER 5: REFERENCES

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